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13. ABSTRACT *(Maximum 200 Words)*

Autocrine motility factor (AMF) is expressed by human breast cancer cells, such as MCF7 where its expression is stimulated by heregulin. Tumor cells constitutively secreting mouse AMF caused periosteal new bone formation in two different models of metastasis-an osteoblastic response similar to what is found with about 15% of breast cancers metastatic to bone. Whenever serum AMF concentrations were significantly increased, the animals displayed tumor-associated weight loss (cachexia), a major cause of morbidity and mortality in advanced disease. The effects of AMF on bone were independent of PTHrP, which plays a central role in osteolytic bone metastases. We also have determined the clearance rate of AMF from the mouse circulation. The species-specific effects of AMF remain little understood; so we undertook to clarify the stucture:function relationships involved. We cloned, sequenced and expressed rabbit AMF, for which the x-ray crystallographic data were already partially solved. These results have been published. We also developed a recombinant protein expression system for the mouse and human factors, which we now prepare in 100mg batches. The human factor has been crystallized and the x-ray structure solved to 1.8Å.

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INTRODUCTION

Recent studies show that breast cancer-secreted proteins play a causal role in the initiation and progression of bone metastases by stimulating osteoclasts. A number of tumor-secreted factors enhance invasion and metastasis, but only one to date has been shown to cause specific metastasis to bone: parathyroid hormone-related protein (PTHrP, a potent stimulator of osteoclastic bone resorption), although other factors secreted by breast cancer cells must also act, either independently or in concert with PTHrP, to increase osteolytic metastases. **Autocrine motility factor** (AMF) may also play a important role in metastasis to bone. It is secreted by breast cancer cells and is a known marker of metastasis. We originally found that it is a potent stimulator of osteoclast formation. We tested the role of AMF in the formation of bone metastasis by human breast cancer cells with 3 original hypotheses and experimental aims: 1) **AMF is secreted from breast cancer cells and regulated by factors released from bone matrix**. MCF7 and MDA-MB-231 human breast cancer cell lines make AMF, but neither expression of the factor by breast cancers nor regulation of its secretion has been widely studied. We tested ten standard breast cancer cell lines for AMF expression and for regulation of secretion by known bone matrix factors, including IGFs 1 & 2, TGF-β (which increases PTHrP), FGFs 1 & 2, and BMP2. We have found no factors which regulate the release of the protein from cells. 2) **Secretion of AMF stimulates bone resorption and osteolytic metastases**. Since AMF increases the numbers of osteoclasts, it should enhance bone resorption and therefore metastases to bone. We compared the formation of bone metastasis by cells known to overexpress AMF relative to control cells in an animal model. We found that in vivo AMF caused periosteal new bone formation but did not stimulate osteolysis. Thus, our original 3rd specific Aim turned out to be incorrect, but we did find important actions of AMF in bone metastases.3) **AMF secretion enhances osteolytic metastasis due to PTHrP**. PTHrP is a well-characterized factor which stimulates bone resorption. We compared in an animal model the formation of bone metastases by breast cancer cells secreting PTHrP alone and cells secreting AMF and PTHrP together. AMF did not increase osteolysis in these experiments. However, we did find that systemic elevation of plasma AMF resulted in cachexia in experimental animals. This important finding has formed the basis of experiments added to the original proposal and which are being carried out during a one year, no-cost extension of our original work.

We have presented this progress report as a detailed summary of all the work accomplished, relative to the original 16 tasks encompassed by the Statement of Work. These are referenced to the original 3 hypotheses/specific aims, which are stated in bold type above, in their original form. We plan to provide a supplement next year to this report. Together they will form the final report for this project.

BODY

First, we reproduce the original, approved Statement of Work page and the attendant Specific Aims and follow this with a report on the disposition [as of June 2001] of each of the task proposed. All new data figures are provided within the appended material:

STATEMENT OF WORK:

Task 1: Grow 10 breast cancer cell lines under 8 different conditions, harvest conditioned media and determine cell number (months 1-12, year 1).

Task 2: Assay AMF and protein concentrations in samples from task 1 (year 2, months 1,2)

Task 3: Determine growth curves of cells for task 4 *in vivo* (10 mice) and *in vitro* (year 1, months 1- 4).

Task 4: Perform animal experiment (20 mice) with CHO & CHO-IC6 cells (year 1, months 5-12)

Task 5: Perform histology and histomorphometry of animals from task 4 (year 2, months 1-12).

Task 6: Transform and select PTHrP-overexpressing clone of CHO-IC6 (year 1 months 6-12).

Task 7: Repeat task 3 (20 mice) for cells from task 6 (year 1, months 1-6)

Task 8: Repeat task 4 (40 mice) for cells from task 6 (year 1, month 7- year 2, month 2).

Task 9: Repeat task 5 for cells from task 6 (year 2, month 3- year 3, month 3).

Task 10: Construct amplifiable AMF expression DNA (year 1, months 1-3).

Task 11: Transfect MDA-MB-231 cells with AMF cDNA and select stable overexpressing cell line (year 1, month 3- year 2, month 6).

Task 12: Repeat task 3 (10 mice)of cells from task 11 (year 2, months 7-12)

Task 13: Repeat task 4 (20 mice) for cells from task 11 (year 3, months 1- 5).

Task 14: Repeat task 5 for cells from task 11 (year 3, months 6-12).

Task 15: Carry out detailed statistical analysis of data from animal experiments (each year months 10-12).

Task 16: Prepare data for publication, including figures, and slides (each year months 10-12).

1) **AMF is secreted from breast cancer cells and regulated by factors released from bone matrix**. MCF7[12] and MDA-MB-231 human breast cancer cell lines make AMF, but neither expression of the factor by breast cancers nor regulation of its secretion has been widely studied. We will screen ten standard breast cancer cell lines for AMF expression and for regulation of its secretion by known bone matrix factors: IGFs 1 & 2, FGFs 1 & 2, PDGF, TGF-β, and BMP2.

2) **Secretion of AMF stimulates bone resorption and osteolytic metastases**. Since AMF increases the numbers of osteoclasts, it should enhance bone resorption and therefore metastases to bone. We will compare the formation of bone metastasis by cells known to overexpress AMF but

not other factors active on bone, relative to control cells, in an animal model in which these cells metastasize to bone. This experiment will test whether AMF alone is sufficient to cause osteolytic metastases.

3) **AMF secretion enhances osteolytic metastasis due to the well-characterized resorptive factor, PTHrP**. We will compare the formation of bone metastases by MDA-MB-231 cells (which form osteolytic metastasis dependent on their secretion of PTHrP and secrete moderate amounts of AMF) with transfected MDA-MB-231 cells secreting high levels of AMF. PTHrP alone causes osteolytic metastasis, but its effects can be potentiated by other factors which stimulate osteoclasts. This experiment will test whether tumor-secreted AMF can increase osteolytic metastases in the presence of PTHrP.

Tasks 1&2: *Successfully Completed.* The data were provided as Figure 6 of the 1999 progress report. In addition we tested a large number of conditions, including the peptide growth factors originally listed in the proposal, and in addition a variety of lectins which have actions to increase secretion of neuroleukin (Gurney et al, 1986). Neuroleukin is one a variety of alternative names for autocrine motility factor. None of the factors tested had a significant effect on secretion of AMF from breast cancer cells. Since the submission of the original application, no significant progress has been made in the field on the still-unknown mechanism by which a variety of proteins, such as AMF, fibroblast growth factors 1 & 2, and others are post-synthetically secreted from the cytoplasm of cells in the absence of cell lysis. Thus, although activation of the *her2/neu* receptor on breast cancer cells increases mRNA for intracellular AMF (Talukder et al, 2000), we still have no idea how to manipulate secretion of the active factor from cells, either in vitro or in vivo. We have thus not pursued this intractable issue further.

Tasks 3-5: *Successfully Completed.* The data were provided in the 2000 progress report, Figures 1-9. These data are ready for submission for publication. Original Specific Aim 2, above, predicted that in vivo AMF would function as an osteolytic factor. We found, however, that it stimulated periosteal new bone formation and, in the absence of osteolytic metastases, caused cachexia - a subject which is discussed further below.

Tasks 6-9: *Successfully Completed.* We found, after the completion of tasks 3-5, that basic CHO cells, both CHO-K1 and CHO-1C6, expressed modest levels of PTHrP [data provided in the 2000 progress report, Figure 10.] These levels are comparable to the MDA-MB-231 cell line, the standard model of osteolytic bone metastasis due to breast cancer (Yin et al, 1999; Chirgwin & Guise, 2000). Thus, unintentionally, tasks 6-9 were performed within the experiments for tasks 3-5. Publication of these results will therefore be included within the publication resulting from tasks 3-5. AMF overexpression did not increase osteolytic metastases in the presence of PTHrP, as originally hypothesized [Specific Aim 3, above].

Task 10: *Successfully Completed.* We have constructed a series of amplifiable expression DNAs for AMF and a series of other nonclassically secreted proteins, including FGF2 and clotting factor XIII A chain transglutaminase. These resulted in nonclassical secretion of the expressed

proteins from transiently transfected cells.

Task 11: *Unsuccessful to date*. We expended a great deal of effort to establish stable cell lines which would continue to secrete transfected protein (We tested all 3 described in the previous paragraph). In every case stable cell clones failed to maintain nonclassical secretion. We spent one year carrying out control experiments with step-wise gene amplification (Schimke, 1984; Bendig, 1988) using the mouse dihydrofolate reductase (dhfr) cassette and selection for increasing levels of resistance to methotrexate (mtx). This method was used by Gurney to create the CHO cell line CHO-1C6 ,although no data on the amplification or selection have every been published. dhfr$^-$ CHO has been the standard cell line for methotrexate-resistant gene amplification, and it may be that breast cancer cells are not amenable to the standard gene amplification protocols in vitro. We have developed an alternate strategy, which is being carried out in the continuation year and is described in detail below.

Tasks 12-14: *Not done*. These tasks depend on task 11. However, the results of the successful task 3-9 have defined the consequences for bone metastasis of PTHrP +/- AMF and render these tasks no longer essential or of central importance. We intend to complete them, although probably after the expiration of the present funding, if we are able to complete the alternative task 11, described below.

Tasks 15-16: *Completed, as well as ongoing*. See previous progress reports as well as Reportable Outcomes, below.

Alternative approach to unsuccessful task 11, presently underway:

Task 11*alt*: As described above, dhfr/methotrexate [mtx] gene amplification is cumbersome and of uncertain outcome (Benndig, 1988; Fann et al, 2000; Kim et al, 2001). In recent years an alternative system has been developed, which is based on the ability of cloned glutamine synthetase [QS] protein to confer resistance to methionine sulfoximine [msx] (Cockett et al, 1990). This system permits much more efficient gene amplification, with only one or two rounds of amplification necessary, as opposed to the year+ of amplification needed with dhfr/mtx. The molecular basis of dhfr and QS amplification (when many other selectable markers do not provide the ability to step-wise amplify co-transfected DNAs) is unclear; however the QS system has been widely used in the biotechnology industry for the recombinant manufacture of humanized monoclonal antibodies expressed in mammalian tissue culture. The QS system is commercially available under license from the Lonza Corporation in Switzerland. However, the intellectual property agreement demanded by this company was so extensive that the University of Texas was unwilling to agree to their conditions after about 6 months of negotiation. Since then we have isolated, sequenced, and expressed a full length human QS cDNA. We are presently testing the ability of this DNA to confer resistance against 20micromolar msx into stably transfected MDA-MB-231 breast cancer cells. If successful, we will resume task 11 using this DNA, pcDNA3neo-hQS as a coselectable marker for gene amplification of mouse and human AMF proteins in transfected breast cancer cells.

Additional tasks not originally proposed but completed (identified by subject rather than creation of new task numbers):

1) Identification of rabbit AMF. Much of the original work on this protein, under one of its alternate names, phosphoglucose isomerase, was done on material isolated from rabbit muscle, because of the similar properties of rabbit and human proteins [e.g., Le et al, 2000]. However, cloning, sequencing, and expression of the rabbit cDNA protein had not been reported; so we undertook this project. The successful results have been published (Li & Chirgwin, 2000) and were included with the 2000 progress report.

2) X-ray crystal structure of human AMF. The past year saw the publication of the rabbit crystal structure (Jeffery et al, 2000, 2001). At the same time we undertook to solve the structure of the human protein. We cloned and expressed human AMF and purified the protein in sufficient quantity for our collaborator, Christopher Davies, then at University of Sussex, UK, and now at the Medical University of South Carolina, rapidly to crystallize and then solve the structure. The extensive results of this collaboration were recently published (Read et al, 2001) and a copy is appended. Our own data show important biological activities of AMF in cancer in vivo on bone and on cachexia, which are in addition to the numerous roles for the protein demonstrated in vitro by us and others (extensively discussed in the original application and previous progress reports). In addition, data published last week (Funasaka et al, 2001) demonstrate that AMF can stimulate angiogenesis in vitro and in vivo. These data reinforce the physiological importance of AMF in cancer. Future studies of this protein can now be guided by detailed x-ray structures of the protein. In particular the human structure was solved to 1.6Å, permitting very detailed analysis. The high resolution permits identification of glutamic acid [E] 357 as the residue directly responsible for isomerization in the phosphoglucose isomerase reaction in the glycolytic pathway inside the cell. The identification of E357 has been confirmed by inhibitor studies with the rabbit protein (Jeffery et al, 2001). These data are important because of results suggestive that isomerase activity may be necessary for cytokine activity. Funasaka et al (2001) report that erythrose 4-phosphate, an isomerase competitive inhibitor, decreased AMF-induced angiogenesis. High doses of the sugar phosphate were required (0.5mg/mouse), so non-specific effects are not easily excluded. A more definitive assessment of the relationship of isomerase to AMF activities could be carried out by active site mutagenesis of residue E357, which is now possible from the crystal structures.

3) Role of AMF in cachexia. Many of these data were included in the previous, year 2000, progress report. At that time, we were not fully aware of the importance of these findings. We have developed this important direction and recently submitted a new Idea proposal to the Army Breast Cancer program. The writing of this proposal has interfered with the timely submission of this progress report. A copy of the scientific sections of this proposal, including the relevant preliminary data, are included with the appended material.

4) Methods for recombinant AMF expression. These are summarized in Li & Chirgwin (2000) and Read et al (2001). We have expended substantial time on these methods, although we spare the reviewers copies of the large number of protein gels generated. We found that somewhat

different conditions and even E coli host strains are necessary for the most efficient expression of the rabbit, mouse, and human proteins. We have purified all of these to apparent homogeneity and shown them to be free of bacterial endotoxin contamination-which would interfere with the biological assays of AMF-like activities. These factors retain isomerase activity and bioactivity on bone cells in vitro. We have compared the isomerase kinetic activities for the recombinant proteins with the rabbit protein conventionally purified from rabbit muscle.These data are being prepared for publication and provide the necessary proof that the bacterially expressed proteins are essentially indistinguishable from the mammalian versions.

5) Monoclonal antibodies against human AMF. These were prepared against recombinant human AMF. Six mouse IgG mAbs have been characterized in detail. Although they do not discriminate between mouse and human AMFs, they have substantial utility as possible inhibitors of the receptor-mediated biological activities of tumor-secreted AMF. Detailed information about these mAbs and their future applications is included within our recently submitted Army Breast Cancer Program Idea proposal, a copy of which is included in the appended material

Additional tasks not originally proposed but ongoing during year 4 (identified by subject rather than creation of new task numbers):

6) Molecular basis of species-specific binding of AMF to mammalian cells. Our data with biological actions of AMF on bone cells (included in the previous progress reports) identified an approximately 100-fold species preference of mouse cells for mouse AMF and human cells for human AMF. Such data have not been reported by others, who have not had access to the mouse factor, which we originally purified from the CHO-1C6 cell line, but are now expressing in E. coli as we previously described for the rabbit protein (Li & Chirgwin, 2000).

7) X-ray crystal structure of mouse AMF. In fashion exactly parallel to that which we used last year to solve the human crystal structure, we have cloned, expressed, purified, and crystallized mouse AMF. This protein also has isomerase activity and is active on mouse bone cell cultures. Dr. Christopher Davies is presently working on the crystal structure.

KEY RESEARCH ACCOMPLISHMENTS (in year 3)

- Pure recombinant AMF produced for 3 species
- Human AMF 3-D structure solved and published
- New model of cancer-induced cachexia developed
- Interspecific mouse:human chimeric AMFs made
- Catalytic site and role of glutamate 357 identified
- Crystal structure for mouse AMF begun
- New gene amplification system with human glutamine synthetase made
- Full-length mouse AMF receptor cDNA cloned
- Monoclonal mouse IgG antibodies against human AMF produced

REPORTABLE OUTCOMES

Three publications:

Chirgwin JM, Guise TA (2000). Molecular mechanisms of tumor-bone interactions in osteolytic metastases. *Crit Rev Eukaryot Gene Expr* **10**:159-178.

Wang F, Duan R, Chirgwin JM, Safe SH (2000). Transcriptional activation of cathepsin D gene expression by growth factors. *J Mol Endocrinol* **24**:193-202. [Not on AMF but the subject of a previous, unfunded, Army Breast Cancer Idea proposal by the P.I.]

Read J, Pearce J, Li X, Muirhead H, Chirgwin J, Davies C (2001). The crystal structure of human phosphoglucose isomerase at 1.6Å resolution: implications for catalytic mechanism, cytokine activity and haemolytic anaemia. *J Mol Biol* **309**:447-463.

CONCLUSIONS

We have completed the main original goal of this project and identified the actions both in vitro (stimulation of osteoclastogenesis) and in vivo (stimulation of periosteal new bone formation) of AMF in breast cancer bone metastases. In addition we discovered an unexpected effect of AMF to cause the paraneoplastic syndrome of cancer cachexia, a subject of major clinical importance. This syndrome is the focus of our future work on AMF. To this end we devoted most of our work in the 03 year to developing the necessary molecular tools for this future direction. We developed the methods for cloning, expressing, and purifying large quantities of mammalian and interspecies chimeric AMF proteins. This culminated in the solution of the crystal structure of human AMF to 1.6angstroms (Read et al, 2001). Also during 2001 data were published (Funasaka et al, 2001) that AMF is an angiogenic agent. Angiogenesis is a major focus of therapies aimed at inhibiting tumor growth and metastasis. Better characterized angiogenic agents, such as VEGF, contribute to osteolytic bone metastases (Chirgwin & Guise, 2001).

AMF continues to grow in importance as its many roles in contributing to the phenotypes of breast cancer cells are realized. Our work has shown several new roles and has supplied important molecular tools, included expressed proteins, mutant proteins, 3-D structure, and monoclonal antibodies, for continuing progress directed against this clinically important factor.

Short-Term Future Directions. We are commencing these experiments, which are summarized under 1-6) above within the period of the 1 year no-cost extension which we have requested to extend the original three years of the proposal. This work was submitted to the DoD as a Breast Cancer Idea grant 6/01. A copy of this proposal is included in the appended material:

TUMOR-SECRETED AMF: CAUSAL ROLE IN AN ANIMAL MODEL OF CACHEXIA

Background: **Autocrine motility factor** (AMF) is expressed by human breast cancer cells, such as MCF7, where its mRNA is stimulated by heregulin. Also known as phosphoglucose isomerase [PGI] and neuroleukin, it has been used as a serum marker of metastatic breast cancer for 50 years. We recently found that tumor cells constitutively secreting AMF caused periosteal new bone formation in two different models of bone metastasis. Animals with significantly increased serum AMF concentrations displayed tumor-associated weight loss (cachexia), a major cause of morbidity and mortality in advanced disease. The cell line engineered to secrete mouse AMF, when grown as an intramuscular tumor, caused severe cachexia in mice, while control tumors were larger but caused no cachexia. This provides strong evidence that **AMF is a novel tumor cachectic factor**: a role consistent with the extensive clinical literature describing AMF/PGI as a serum marker of

advanced metastatic disease.

AMF binds to cell surface receptors. We found that AMF induced expression in bone marrow stromal cells of RANK ligand, a potent bone-resorbing factor. The responses displayed a 100-fold species-specificity for mouse versus human AMFs. Despite its clear importance in breast cancer, the mechanisms of action of AMF via receptor binding are under-investigated. AMF is the secreted form of an intracellular glycolytic enzyme, phosphoglucose isomerase, but the relationship between extracellular and intracellular forms of the protein is controversial.

Objectives & Specific Aims: We propose a series of specific aims to answer five outstanding questions about the induction of cachexia by AMF and the binding of AMF ligand to its receptor:

1) **Is recombinant AMF protein sufficient to cause cachexia in vivo?** Recombinant mouse AMF will be delivered continuously to mice via mini-pumps. The first experiment will determine the dosage to achieve blood levels equivalent to those seen in mice with tumor-induced cachexia. AMF may act through inflammatory cytokines. We will test for increases in the cachexia-associated cytokines, IL-1, IL-6, IFN-γ, and TNFα, in AMF-treated mice.

2) **Is isomerase catalytic activity required for AMF-induced cachexia?** Inside the cell PGI interconverts glucose and fructose 6-phosphates in the glycolytic pathway. It is unclear whether this enzymatic activity is necessary for the extracellular cytokine functions of the protein. We will make 2 mutants of AMF/PGI: E357A, which lacks the side-chain responsible for catalytic proton transfer, and S209T211,214,217/4A, which eliminates the groups needed to bind the phosphate group of the substrate. Experiments will be guided by our 1.6Å x-ray structure of the human enzyme. Mutant proteins will be assayed in vitro for isomerase activity and receptor binding and in vivo for ability to cause cachexia.

3) **Are the cachectic actions of AMF species-specific?** Bone cells in vitro show a 100-fold preference for AMF of the same species when mouse and human are compared- predicting that much higher concentrations of human AMF than mouse AMF will be required to cause cachexia in mice. We will test this prediction as in Aim 1.

4) **Does AMF act via a high-affinity cell-surface receptor?** AMF shows a bell-shaped dose response curve in vitro and in vivo, suggesting an activation mechanism in which receptor is dimerized by low, pM concentrations of dimeric ligand. We will test this mechanism by biochemical binding studies in vitro using [^{125}I] mouse and human AMF ligands and mouse and human cell lines.

5) **Can cachexia be alleviated by treatment with monoclonal antibodies which prevent AMF binding to its receptor?** We have raised a panel of monoclonal antibodies [mAbs] against recombinant AMF/PGI. We will test the individual antibodies for their ability to inhibit ligand binding as in Aim 4. Blocking antibodies will be tested in vivo, as time permits, for their ability to decrease cachexia induced by recombinant AMF/PGI as in Aim 1.

Study Design: Various forms of AMF will be expressed in *Escherichia coli* and purified to homogeneity. They will be tested for binding to cells and by enzymatic assay in vitro. AMFs will be continuously infused into mice via implanted Alzet mini-pumps. AMF concentrations will be assayed in the circulation and cachectic actions determined from body weight and histology of kidney, spleen and liver after sacrifice.

Relevance: All of the technology for the proposed work is proven and available in the P.I.'s laboratory. Recombinant proteins, including a high resolution crystal structure of human AMF, have

been generated, as well as a reproducible animal model for tumor-induced cachexia. Actions of AMF have previously not been studied in vivo. The binding of AMF to its receptor offers a target for development of therapeutics, such as the mAbs to be tested in Aim 5, to decrease the morbidity and mortality caused by advanced, metastatic breast cancer. The work proposed will critically test an important, novel pathological role for AMF in vivo.

Long-Term Future Directions. These are beyond the scope of the original proposal or the experimental aims which we have added in progress. However, these represent the outlines of grant proposal(s) which we anticipate having ready for submission within the next 24 months:

IDENTIFICATION OF RECEPTOR FOR TUMOR-SECRETED AMF

Autocrine motility factor (AMF) is secreted by many human cancer cells. Its expression by MCF7 cells is stimulated by heregulin. It stimulates tumor cell motility and has been a serum marker of metastatic breast cancer for 50 years. We recently found that tumor cells constitutively secreting AMF caused periosteal new bone formation in two different models of bone metastasis -a response similar to what is found with 15% of breast cancers metastatic to bone. Animals with significantly increased serum AMF concentrations displayed tumor-associated weight loss (cachexia), a major cause of morbidity and mortality in advanced disease. AMF induced stromal expression of RANK ligand, a potent stimulator of bone remodeling. Responses showed a 100X species specificity for mouse versus human AMFs.

Tumor-produced AMF exerts its effects by binding to a high affinity receptor on cells. A receptor cDNA was reported nearly a decade ago, but the described protein sequence was wrong. There is no convincing evidence that the real receptor for AMF has been cloned. The real receptor is an important target for therapeutic intervention in metastatic cancer. It is imperative the true receptor for AMF be identified. We propose **3 specific aims**:

1) Do mouse and human cells express high-affinity species-specific receptors for AMF? Mouse and human recombinant AMF proteins will be labeled and used in Scatchard binding analysis to determine the biochemical parameters of ligand binding (affinity constant and number of binding sites per cell) to several standard cell lines. We expect that the same-species binding affinities will be 100X higher than the cross-species bindings.

2) Does the sequence published as the AMFR encode a protein which binds AMF ligand? The answer to this question is almost certain to be no. We have isolated, in a mammalian expression vector, a full-length cDNA corresponding to the published receptor. This will be transiently expressed in human 293 cells, which will then be assayed for receptor at the cell surface and for changes in number and affinity of binding sites for labeled mAMF.

3) What is the best cell source for expression cloning of the real receptors for AMF? The ligand-binding assay from Aim 1) will identifying the true receptor. We will screen for tissue/cell sources abundantly expressing AMF binding activity. The best will be used to prepare a mammalian expression library, from which pools of clones will be screened for high binding of labeled AMF. Pools will be sub-fractionated of identify the real receptor.

Long Term Goals: We have a series of mutants of mouse and human AMF with which to probe the relation between ligand structure and receptor-mediated functions. We have solved the

human AMF structure to 1.6Å and collected equivalent data for the mouse and have demonstrated novel biological responses to AMF *in vivo* and *in vitro*. Future progress is absolutely dependent on the availability of the cloned receptor. We submitted a proposal to the DoD to characterize the ligand:receptor interactions of AMF before suspecting that the published receptor was incorrect. A planned R01 application to the NCI cannot be submitted until the uncertainty about the identity of the receptor is rapidly resolved. The proposed work will enable us to propose the molecular cloning of the correct receptor, to be followed by the characterization of it is functions and signaling in mammalian cells.

Significance: AMF is secreted by a variety of breast and other cancer cells. It is a well-established marker of metastatic disease, has a variety of effects on normal cells, and may play a central role in tumor-produced cachexia and other paraneoplastic syndromes. Since AMF is not a normal extracellular protein in adults, its receptor offers an attractive target for therapeutic intervention in metastatic disease. The work proposed will provide the tools to screen for effective drugs to block the pathological effects of AMF secreted by tumor cells.

RECEPTOR-MEDIATED ACTIONS OF TUMOR-SECRETED AMF

Autocrine motility factor (AMF) is expressed by human breast cancer cells, such as MCF7, where its expression is stimulated by heregulin. Also known as phosphoglucose isomerase and neuroleukin, it has been used as a serum marker of metastatic breast cancer for 50 years. We recently found that tumor cells constitutively secreting AMF caused periosteal new bone formation in two different models of bone metastasis -a response similar to what is found with about 15% of breast cancers metastatic to bone. Animals with significantly increased serum AMF concentrations displayed tumor-associated weight loss (cachexia), a major cause of morbidity and mortality in advanced disease. AMF induced stromal cell expression of RANK ligand, a potent bone-resorbing factor. These responses displayed a 100-fold species specificity for mouse versus human AMFs.

AMF may exert its extracellular effects by binding to a recently-described. novel member of the G-protein coupled, seven-transmembrane domain class of receptors, and then activating protein kinase C and subsequent nuclear transcription. Despite its clear importance in breast cancer, the mechanisms of action of AMF through its receptor are uninvestigated. Although AMF is the secreted form of an intracellular glycolytic enzyme, phosphoglucose isomerase, the relationship between extracellular and intracellular forms of the protein is controversial. We propose a series of specific aims to answer five outstanding questions:

1) **1) Is AMF protein in fact a high-affinity, species-specific ligand for its putative receptor?** Cloned mouse and human AMF proteins will be labeled and used to determine binding constants to cells stably transfected with plasmids expressing the cloned mouse and human receptors. Species-specificity and its molecular basis will be determined with a series of chimeric AMF proteins generated by switching segments of mouse and human coding sequences and expressing the recombinant proteins in *E. coli*.
2) **Is isomerase catalytic activity required for receptor responses?** It has been claimed

that a bacterial phosphoglucose isomerase has AMF activity and that mammalian AMF function requires isomerase activity, but the data are unconvincing. We will purify and assay two mutants of AMF: E357A, which lacks the glutamate responsible for catalytic activity, and S209T211,214,217/4A, which eliminates the groups needed to bind the phosphate group of the substrate. These experiments will be guided by the x-ray structure of the recombinant human enzyme, which we have recently resolved to 1.6Å. The mutant proteins will be tested in 3 assays for: a) isomerase activity, b) RANK ligand induction, and c) ability to cause monocytic differentiation *in vitro*.

3) Does the receptor activate PKC-responsive gene transcription? Cells will be stably transfected with PKC-responsive reporters (AP-1-containing *fos*-luciferase or the human interleukin 1β promoter-luciferase) plus the AMF receptor expression DNAs. The cells will be treated with purified AMF ligands and the dose- responses of the luciferase reporter readouts determined.

4) Is signaling initiated by a ligand-induced receptor dimerization mechanism? AMF shows a bell-shaped dose response curve *in vitro* and *in vivo*, suggesting an activation mechanism in which the receptor is dimerized by low concentrations [pM] of the ligand (an obligate dimer). At saturating concentrations, each receptor is bound by a separate AMF molecule; so receptor dimerization is opposed. This mechanism will be tested by fusing the ligand-binding N-terminal half of the receptor to the cytoplasmic domain of the erythropoietin receptor. This is a well-established system to study ligand-induced dimerization. Ligand induced dimerization of the EpoR cytoplasmic domains can be assessed by its ability to confer IL-3 independent survival to the transfected Ba/F3 cell line.

5) Does receptor activation induce transcription of genes associated with cachexia? AMF causes differentiation of monocytic cell lines *in vitro*, as does stimulation of PKC signaling -the known target of AMF receptor activation. A consequence of such activation is the induction of interleukin 1 secretion by monocytes. IL-1 is a potent inflammatory factor, which suggests an important causal relationship between elevated serum AMF levels and tumor-associated cachexia. We will test the ability of pure, recombinant AMF protein to induce the expression from monocytes of cachexia-associated cytokines, such as IL-1, IL-6, and TNFα.

G-protein coupled, seven-transmembrane domain receptors offer excellent targets for the development of pharmacological inhibitors. The work proposed will provide all of the tools to screen for effective drugs to block the effects of AMF secreted by breast cancer cells.

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APPENDICES [4 items]

Three publications:

Chirgwin JM, Guise TA (2000). Molecular mechanisms of tumor-bone interactions in osteolytic metastases. *Crit Rev Eukaryot Gene Expr* **10**:159-178.

Wang F, Duan R, Chirgwin JM, Safe SH (2000). Transcriptional activation of cathepsin D gene expression by growth factors. *J Mol Endocrinol* **24**:193-202. [Not on AMF but the subject of a previous, unfunded, Army Breast Cancer Idea proposal by the P.I.]

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One Army Breast Cancer Program Idea Application, submitted 6/01:

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Molecular Mechanisms of Tumor–Bone Interactions in Osteolytic Metastases

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ABSTRACT: In patients with advanced disease, several cancer types frequently metastasize to the skeleton, where they cause bone destruction. Osteolytic metastases are incurable and cause pain, hypercalcemia, fracture, and nerve compression syndromes. It was proposed over a century ago that certain cancers, such as that of the breast, preferentially metastasize to the favorable microenvironment provided by bone. Bone matrix is a rich store of immobilized growth factors that are released during bone resorption. Histological analysis of osteolytic bone metastases indicates that the bone destruction is mediated by the osteoclast rather than directly by the tumor cells. These observations suggest a vicious cycle driving the formation of osteolytic metastases: tumor cells secrete factors stimulating osteoclasts through adjacent bone marrow stromal cells; osteoclastic resorption in turn releases growth factors from the bone matrix; finally, locally released growth factors activate the tumor cells. This vicious cycle model has now been confirmed at the molecular level. In particular, transforming growth factor β (TGFβ) is abundant in bone matrix and released as a consequence of osteoclastic bone resorption. Bone-derived TGFβ plays an integral role in promoting the development and progression of osteolytic bone metastases by inducing tumor production of parathyroid hormone-related protein (PTHrP), a known stimulator of osteoclastic bone resorption. In breast cancer cells TGFβ appears to stimulate PTHrP secretion by a posttranscriptional mechanism through both Smad and p38 mitogen activated protein (MAP) kinase signaling pathways. Osteolytic metastases can be suppressed *in vivo* by inhibition of bone resorption, blockade of TGFβ signaling in tumor cells, and by neutralization of PTHrP. Other factors released from bone matrix may also act on tumor cells in bone, which in turn may produce other factors that stimulate bone resorption, following the vicious cycle paradigm established for TGFβ and PTHrP. An understanding at the molecular level of the mechanisms of osteolytic metastasis will result in more effective therapies for this devastating complication of cancer.

KEY WORDS: PTHrP, TGF-β, osteolysis, bone metastasis, breast cancer.

I. INTRODUCTION

Metastasis of cancers to bone often causes bone destruction, or osteolysis, resulting in bone pain, fracture, hypercalcemia, and nerve compression. Several solid tumor types, such as prostate, lung, renal, and thyroid, are associated with osteolytic lesions, but breast cancer is the most common. Nearly 70% of patients dying of breast cancer have bone metastases (Coleman and Rubens, 1987). Patients with cancer may survive several years with bone metastases so it is important to understand the process to improve therapy and prevention. This article highlights recent developments in our understanding of the molecular mechanisms of osteolytic bone metastases.

A model explaining the association of breast cancer with skeletal morbidity was proposed in 1889 when Stephen Paget observed that "in a cancer of the breast the bones suffer in a special way, which cannot be explained by any theory of embolism alone." Breast cancer has a marked affinity to grow in bone. The mechanisms underlying this osteotropism are complex and involve

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unique characteristics of both the breast cancer cells and the bone to which tumors metastasize. Paget proposed the "seed and soil" hypothesis to explain this phenomenon. "When a plant goes to seed, its seeds are carried in all directions; but they can only grow if they fall on congenial soil" (Paget, 1889). The microenvironment of the organ to which the cancer cells metastasize provides a fertile soil on which the cancer cells (seeds) grow (Guise and Mundy, 1998). This century-old concept remains a basic principle of cancer metastasis, guiding current progress in understanding the molecules produced by bone and by tumor cells that drive the vicious cycle.

II. PATHOGENESIS OF OSTEOLYTIC BREAST CANCER METASTASES TO BONE

A. Role of the Bone Microenvironment

Bone is unique among target tissues affected by cancer because it is being continually remodeled under the influence of systemic hormones and local bone-derived growth factors. Bone consists of two physically and biologically distinctive structures. The outer cortical bone is hard mineralized matrix in which cellular and metabolic activities are relatively low. Cortical bone makes up 85% of the total bone in the body and is most abundant in the long bones of the appendicular skeleton. The volume of cortical bone is regulated by the formation of periosteal bone, by remodeling within Haversian systems, and by endosteal bone resorption. Cancellous or trabecular bone constitutes the remaining 15% of the skeleton, and is most abundant in the vertebral bodies. The adult skeleton is in a dynamic state because the coordinated actions of osteoclasts and osteoblasts on trabecular surfaces and in Haversian systems result in continual bone resorption and formation. The organic component of bone matrix forms a large storehouse for immobilized growth-regulating factors (Hauschka et al., 1986; Mohan and Baylink, 1991). These include insulin-like growth factors (IGFs) 1 and 2, TGFβ, fibroblast growth factors (FGFs) 1 and 2, platelet-derived growth factors (PDGF), and bone morphogenetic proteins (BMPs). This list is not comprehensive, because more recently discovered factors may also be stored in bone but have not yet been assayed for their presence in matrix. All of the identified factors have effects on various cancer cell types. However, the factors must be released in soluble form from the bone matrix before they can bind to their receptors on tumor cells. Such growth factors are released from the bone matrix as a result of osteoclastic bone resorption (Pfeilschifter and Mundy, 1987), a component of the normal remodeling process necessary to maintain the structural integrity of bone.

The inner portion of bone consists of multicellular bone marrow in which hematopoietic stem cells, stromal cells, and immune cells reside. The hematopoietic stem cells have the potential to differentiate into the blood-forming elements and bone-resorbing osteoclasts, whereas the stromal cells support the differentiation of the hematopoietic cells as well as form bone-producing osteoblasts. Cells in the bone marrow, stromal and immune in particular, produce cytokines and growth factors that mediate cell-to-cell interactions in autocrine, paracrine, and/or juxtacrine fashions. Once cancer cells arrest in bone, the high concentrations of growth factors and cytokines in the bone microenvironment provide a fertile soil on which the cells can grow. Furthermore, when the tumor cells stimulate osteoclastic bone resorption, this bone microenvironment is even more enriched with bone-derived growth factors that enhance survival of the cancer and similarly disrupt normal bone remodeling to result in bone destruction. Osteoclastic bone resorption releases locally high concentrations of ionized calcium, in addition to growth factors, into the bone microenvironment, so extracellular calcium could have effects on tumor cells metastatic to bone, as is discussed later in this review.

B. Role of Tumor Cells to Stimulate Osteolysis

Tumor cells abundantly secrete acid and proteolytic enzymes and can destroy bone matrix *in vitro* (Eilon and Mundy, 1978). However, it is

likely that *in vivo*, particularly during the establishment of metastases, bone is resorbed by osteoclasts rather than by tumor cells. Histological analysis and scanning electron microscopy of osteolytic bone metastases indicate that the bone destruction is mediated by the osteoclast (Taube et al., 1994; Boyde et al., 1986). Bisphosphonates, potent inhibitors of bone resorption, significantly reduce skeletal morbidity in patients with advanced breast cancer (Hortobagyi et al., 1996) and reduce metastasis to bone by human breast cancer cells in an animal model (Sasaki et al., 1995).

Osteoclasts are specialized multinucleated cells derived from the same hematopoietic lineage as monocytes and macrophages. They are an infrequent type among the cells lining the surface of bone, but dramatic progress has been made in recent years in understanding the molecular regulation of their formation and activity (Reddy and Roodman, 1998; Martin et al., 1998; Suda et al., 1999). The majority of factors that stimulate bone resorption do so not by acting directly on osteoclasts or their precursors, but by indirect actions on bone marrow stromal cells in the osteoblastic lineage (Reddy and Roodman, 1998). The factors include prostaglandin E2, 1,25dihydroxyvitaminD3, interleukins 6 and 11, and PTH and PTHrP. These molecules converge to stimulate production of a signaling molecule on the stromal cell surface, RANK ligand, which activates receptor activator of NFκB (RANK) on osteoclasts and their precursors (Suda et al., 1999). The effects of RANK ligand are opposed by a soluble binding protein or decoy receptor, osteoprotegerin (Opg), also expressed by bone marrow stromal cells. Pertinent to the molecular mechanisms of osteolytic metastases is experimental evidence that tumor-produced PTHrP mediates osteolysis via increasing RANK ligand and decreasing Opg expression by the osteoblast (Thomas et al., 1999). The functions of RANK ligand and Opg as well as their other names are discussed in detail in a later section.

C. Role of Tumor-Produced PTHrP

Tumor-secreted factors, such as PTHrP, are often responsible for the osteoclast-stimulating activity and bone destruction caused by breast cancer (Bundred et al., 1991,1992; Powell et al., 1991; Vargas et al., 1992; Bouizar et al., 1993; Guise et al., 1996). PTHrP was purified from human lung cancer (Moseley et al., 1987), breast cancer (Burtis et al., 1987), and renal cell carcinoma (Strewler et al., 1987) as a hypercalcemic factor by three groups in 1987 and was cloned shortly thereafter (Suva et al., 1987). PTHrP has 70% amino acid identity to the first 13 amino acids of parathyroid hormone (PTH). Both bind to a common PTH/PTHrP receptor (type I PTH/PTHrP receptor; Abou-Samra et al., 1992). They share similar biological activities (Horiuchi et al., 1987; Kemp et al., 1987). In hypercalcemia of malignancy, tumor-produced PTHrP binds to type I PTH/PTHrP receptors in bone and kidney to cause hypercalcemia, osteoclast-mediated bone resorption, increased nephrogenous cAMP, and phosphate excretion. The PTH-like properties of PTHrP to increase osteoclastic bone resorption and renal tubular calcium reabsorption are responsible for the hypercalcemia.

About 80% of hypercalcemic patients with solid tumors have detectable or increased plasma PTHrP concentrations (Burtis et al., 1990). Approximately 50% of human primary breast cancers express PTHrP (Bundred et al., 1991,1992; Southby et al., 1990). Some of these studies indicate that women with PTHrP-positive tumors are more likely to develop bone metastases (Bundred et al., 1992). However, PTHrP expression by breast cancer at metastatic sites differ significantly from that of the primary site. PTHrP is expressed by greater than 90% of breast cancer metastases to bone compared with only 17% of nonbone metastases or 50% of primary tumors (Powell et al., 1991; Vargas et al., 1992; Southby et al., 1990). These observations suggest two explanations: that PTHrP, as a bone resorbing factor, favors tumor growth in bone or that the bone microenvironment enhances the production of PTHrP. The explanations are not mutually exclusive. Furthermore, interpretation of these clinical findings are limited by the fact that the data on PTHrP expression in metastatic and primary tumor tissue were obtained from different patients. To date, there are no published studies that compare PTHrP expression by the primary and metastatic tumor in the same patient.

Data presented in abstract form add complexity to this issue. A prospective study of over 300 breast cancer patients indicates that those whose primary tumors were PTHrP-negative were more likely to develop bone metastases (Henderson et al., 1999). Tumor tissue from both bone metastases and primary tumor were available from three patients. In these cases, the bone metastases were PTHrP-positive and the respective primary tumors were PTHrP-negative. Data from this small subset of patients suggest that tumor PTHrP expression is enhanced in the bone microenvironment. However, larger numbers will be needed to confirm these observations.

III. EXPERIMENTAL EVIDENCE FOR PTHrP AND TGFβ IN OSTEOLYTIC METASTASES

A. Role of PTHrP

The early clinical studies that suggested a role for PTHrP as a local mediator of bone destruction by metastatic breast cancer provided the rationale for extensive experimental studies in this area. In a mouse model of bone metastases, a neutralizing monoclonal antibody to PTHrP-(1-34) inhibited the development of breast cancer metastases to bone by the human breast cancer cell line, MDA-MB-231, which produces moderate amounts of PTHrP in a nude mouse model (Guise et al., 1996). Histomorphometric analysis of long bones from tumor-bearing mice revealed significantly fewer osteoclasts at the tumor-bone interface and less tumor in animals treated with the PTHrP antibody compared with the controls. Similar results were obtained by Iguchi et al. (1996) with lung cancer cells. Thus, neutralizing the effects of PTHrP decreased not only osteoclastic bone resorption, but also tumor burden in bone. Experimental overexpression of PTHrP by MCF-7 breast cancer cells, which do not express PTHrP and do not cause osteolytic metastases, induced marked bone destruction and increased osteoclast formation, compared to the controls that caused no bone metastases (Thomas et al., 1999).

The data together suggest that tumor production of PTHrP is important for the establishment and progression of osteolytic bone metastases. Unanswered, however, was the possibility that the bone microenvironment enhances the production of PTHrP. The regulation of PTHrP is complex, and factors such as prolactin, epidermal growth factor (EGF), insulin, IGFs 1 and 2, TGF-α, TGF-β, angiotensin II, stretch, and the src protooncogene have been shown to increase expression, whereas glucocorticoids and $1,25(OH)_2D_3$ decrease it (Guise and Mundy, 1998).

B. Role of TGFβ in Osteolysis

As mentioned above, bone matrix is a repository for growth regulatory factors (Hauschka et al., 1986), of which TGFβ is one of the most abundant. Pfeilschifter and Mundy (1987) showed that osteoclastic bone resorption releases the factor in active form. TGFβ enhances production of PTHrP by tumor cells (Southby et al., 1996; Merryman et al., 1994; Kiriyama et al., 1992; Yin et al., 1999a).

Because clinical and laboratory data showed increased PTHrP expression by breast cancer cells in the bone microenvironment, factors known to be present in bone matrix were tested on PTHrP production by human MDA-MB-231 breast cancer cells *in vitro*. Only TGFβ increased PTHrP production by these cells in a dose-dependent fashion (Yin et al., 1999a). Growth factors also abundant in bone, such as FGF-1 and -2, IGF-1 and -2, BMP-2 and PDGF, had no effect on PTHrP secretion by these breast cancer cells. Data from several PTHrP-expressing human tumor types suggest that the major effect of TGFβ to enhance PTHrP production is posttranscriptional, by messenger RNA stabilization (Zakalik et al., 1992; Kiriyama et al., 1993; Merryman et al., 1994; Heath et al., 1995).

TGFβ normally stimulates mesenchymal cell proliferation and extracellular matrix biosynthesis, while inhibiting growth of epithelial cells (Massagué, 1998). These effects are mediated through a heteromeric transmembrane serine-threonine kinase receptor complex. TGFβ binds to a type II receptor dimer, and this complex recruits and phosphorylates a type I receptor dimer, which in turn initiates signal transduction through members of

the Smad protein family. Receptor-regulated Smad proteins (Smad2 and 3) contain a characteristic C-terminal Ser-Ser-X-Ser motif in which the two most terminal serines become phosphorylated in response to TGFβ (Zhang and Derynck, 1999; Massagué, 1998). Recent evidence indicates that the TGFβ-receptor complex also mediates effects via the MAP kinase pathway (Mulder, 2000).

It is now clear that TGFβ has a dual role in malignancy. It inhibits growth of epithelial and some tumor cells, and inactivations or deletions of TGFβ receptors or Smad proteins occur in some carcinomas (Hahn et al., 1996). By contrast, TGFβ can also promote tumorigenesis and invasion in cells that have been transformed (Oft et al., 1998; Welch et al., 1990; Cui et al., 1996). We proposed another role for TGFβ in malignancy: TGFβ mobilized from the bone matrix increased metastasis by stimulating tumor production of PTHrP. This hypothesis was tested using a nude mouse bone metastases model. Transfection of a dominant-negative mutant (TβRIIΔcyt) of the TGFβ type II receptor (Wieser et al., 1993), lacking the kinase domain and which cannot phosphorylate the type IR, made MDA-MB-231 cells unresponsive to TGFβ. TGFβ stimulation of PTHrP production was abrogated. MDA/TβRIIΔcyt cells caused significantly less bone destruction, stimulated fewer osteoclasts, and formed less tumor in bone compared to control cells. Survival was increased in MDA/TβRIIΔcyt-bearing mice. Expression of a constitutively active TGFβ type I receptor [TβRI(T204D)]; Wieser et al., 1995) reversed the dominant-negative blockade, causing increased PTHrP production, marked enhancement of osteolytic bone metastasis, and decreased survival. Introduction of the cDNA for PTHrP, driven by a constitutively active CMV promoter, into the MDA-MB-231 clonal line which already expressed the dominant-negative type II TGFβ receptor, increased PTHrP production and accelerated the development of osteolytic bone metastases (Yin et al., 1999a). Thus, the dominant-negative blockade of TGFβ on MDA-MB-231 cells could be reversed by either a constitutively active TGFβ type I receptor or PTHrP. These experiments demonstrate that both TGFβ-receptor activation and PTHrP are important for the development and progression of osteolytic bone metastases.

C. PTHrP is the Effector of TGFβ in the Pathogenesis of Osteolytic Bone Metastases

The data described in the preceding sections suggest that TGFβ promotes breast cancer metastases to bone through enhanced tumor production of PTHrP. However, they do not exclude other possible effects of TGFβ. To assess the contributions of other effects of TGFβ to promote breast cancer metastases to bone, female nude mice were inoculated with the MDA-MB-231 clonal line that expressed the constitutively active type I TGFβ receptor, TβRI(T204D). The mice were treated with a neutralizing monoclonal antibody directed against PTHrP(1-34) (PTHrP-Ab). Osteolytic lesion area and lesion number were significantly reduced in antibody-treated mice compared to those treated with control IgG. Bone histomorphometry showed significantly smaller tumor area and fewer osteoclasts in tumor-bearing mice treated with PTHrP antibody. The results established that the major effector of TGFβ on the development and progression of bone metastases was PTHrP (Yin et al., 1999a).

D. Role of Smads in Osteolytic Metastases-Tumor Cell Signaling Pathway(s) which Mediate the Effects of TGFβ to Stimulate PTHrP Production

Many, but not all, of the effects of TGFβ are mediated by the Smad signaling pathway (Hocevar et al., 1999). To test if TGFβ stimulated PTHrP production via Smad signaling molecules, MDA-MB-231 cells were transfected with a dominant-negative Smad2 [Smad2(3S-A)], in which 3 C-terminal serines are replaced by alanine. This mutant abolishes TGFβ receptor-dependent phosphorylation (Macias-Silva et al., 1996). Stable clones were unresponsive to TGFβ as assessed by transient transfection with the TGFβ-responsive plasminogen activator inhibitor (PAI) promoter linked to luciferase, 3TPlux. PTHrP production by MDA/Smad2(3S-A) did not increase in response to TGFβ, in contrast to parental MDA-MB-231 cells or stable MDA-MB-231 clones expressing wild-type Smad2 or empty vector (Yin et al., 1999b).

When MDA-MB-231 cells were stably transfected with a series of dominant-negative forms of Smads 2, 3 and 4, PTHrP production in response to TGFβ was reduced but not totally suppressed. When a series of protein kinase inhibitors were tested in a similar experiment, inhibitors of p38 MAP kinase (which is downstream of TAK1, TGFβ-activated kinase) gave similar results. Combined inhibition of Smad and p38 signaling eliminated TGFβ stimulation of PTHrP production as effectively as the dominant negative TGFβ receptor (Käkönen et al., 2000). These results suggest that breast cancer cells use both Smad and p38 signaling pathways downstream of the TGFβ receptor to regulate PTHrP production.

E. Additional Roles of PTHrP and the Type I PTH/PTHrP Receptor

Prostate cancers metastatic to bone result in a predominantly osteoblastic, rather than osteolytic, response. These tumors, however, show frequent expression of PTHrP and can cause osteolytic metastases in animal models (Blomme et al., 1999; Rabbani et al., 1999). Recent results suggest that PTHrP has autocrine growth effects on prostate cancer cells (Dougherty et al., 1999), which have not been observed with breast cancer cell lines such as MDA-MB-231. PTHrP can have effects on expressing cells by at least two distinct mechanisms. The first is an intracrine one, in which clustered basic residues in the PTHrP sequence target the protein to the nucleus (Lam et al., 1999), where it could exert effects on growth by unknown means (Henderson, 1997). A second mechanism is by autocrine signaling through the type I PTH/PTHrP receptor, a seven-transmembrane domain, G-protein coupled molecule that recognizes the amino-terminal regions of PTH and PTHrP and signals through both cyclic AMP and inositol tris-phosphate pathways (Mannstadt et al., 1999). The roles of the type I PTH/PTHrP receptor in osteolytic metastasis have been little studied, but we found that stable transfection of a constitutively activated mutation of this receptor into MDA-MB-231 cells (which do not express the receptor) resulted in decreased basal and TGFβ-stimulated PTHrP production and decreased bone metastases (Rankin et al., 1999). Thus, the role of the type I PTH/PTHrP receptor in the pathogenesis of osteolytic bone metastases remain unclear. It is likely that PTHrP and its receptor may have complex autocrine effects on growth and intracellular signaling of tumor cells in bone. These effects may differ for different tumor types and clearly warrant further investigation.

IV. CELL TYPES INVOLVED IN TUMOR-STIMULATED OSTEOLYSIS

A. Bone Stromal Cells Control Osteoclast Formation and Activity by Expression of RANK Ligand and Osteoprotegerin

Although PTHrP is clearly a potent stimulator of bone resorption, tumor-produced PTHrP may not have direct actions on cells of the osteoclastic lineage. Thomas et al. (1999) showed that a recently identified osteoclast differentiation factor mediates the effects of PTHrP on bone resorption. The stromal cell-expressed RANK ligand, also known as osteoclast differentiation factor (ODF), osteoprotegerin ligand (OPGL), and TRANCE, has been identified. A soluble form of the molecule in combination with macrophage colony-stimulating factor (M-CSF) can generate osteoclasts from hematopoietic cells in the absence of osteoblastic stromal cells (Quinn et al., 1998; Lacey et al., 1998; Yasuda et al., 1998b; Fuller et al., 1998). It was also identified by its ability to induce NFκB and apoptosis of T cells as RANKL and TRANCE, respectively (Anderson et al., 1997; Wong et al., 1997a,b). For consistency, the nomenclature of RANK ligand as indicated by a recent review and consensus (Suda et al., 1999) will be used.

RANK ligand is a member of the TNF family and is a membrane-bound molecule. Opposing the actions of membrane-bound RANK ligand is soluble Opg (Simonet et al., 1997; Yasuda et al., 1998a). Opg is a secreted TNF receptor family member whose overexpression in mice resulted in osteopetrosis (Simonet et al., 1997). Con-

versely, mice deficient in Opg were osteoporotic, consequent to unrestrained osteoclast activity. Confirming the phenotypes of the mice with altered Opg production, recombinant soluble Opg inhibited osteoclast formation in co-cultures of mouse osteoblastic cells and hematopoietic cells (Simonet et al., 1997; Yasuda et al., 1998a). Thus, Opg functions as a decoy receptor for RANK ligand (Simonet et al., 1997; Yasuda et al., 1998a,b). The receptor responsible for signaling RANK ligand biological actions appears to be receptor activator of NFκB, RANK (Anderson et al., 1997). The phenotype of mice homozygous for null alleles of RANK demonstrate that it is the major mediator of the effects of RANK ligand and is essential for normal osteoclast formation (Dougall et al., 1999). RANK signaling also plays an essential role in lymph node formation, whereas its role in osteoclastogenesis normally requires the presence of the permissive factor, M-CSF (Suda et al., 1999). Interleukin (IL) 1 and tumor necrosis factor (TNF) α have RANK-independent effects on osteoclastogenesis (Kobayashi et al., 2000), but the quantitative importance of these pathways is presently unclear and their contributions to bone metastasis are unknown. A variety of organic mediators (prostaglandin E2, 1,25-dihydroxyvitaminD3) and protein factors (PTH, PTHrP, IL-6, IL-11) appear to exert their osteoclastogenic actions primarily via stimulation of RANK ligand expression on the surface of cells in the osteoblast lineage. Nearly all of these conclusions are based on data obtained with mice. The role of the RANK ligand pathway in bone resorption stimulated by IL-1, IL-6, and TNFα may be different in humans compared to mice (Hofbauer et al., 1999).

B. PTHrP Stimulates Osteoclastic Bone Resorption through Stromal Cells and RANK Ligand

Thomas et al. (1999) determined that the breast cancer lines MDA-MB-231, MCF-7, and T47D and primary breast tumor cells did not express RANK ligand but did express Opg and RANK. MCF-7, MDA-MB-231, and T47D cells failed to support osteoclast formation in coculture experiments, consistent with their lack of expression of RANK ligand. MCF-7 cells overexpressing PTHrP were added to cocultures of murine osteoblasts and hematopoietic cells. Osteoclast formation resulted without the addition of osteotropic agents, whereas cocultures with MCF-7 or MCF-7 cells transfected with empty vector required exogenous agents. Similar results were obtained by Chikatsu et al. (2000). When MCF-7 cells overexpressing PTHrP were cultured with murine osteoblasts, osteoblastic RANK ligand mRNA levels were enhanced and osteoblastic Opg mRNA levels diminished, as determined with PCR primers specific for the mouse factors. MCF-7 parental cells had no effect on these mRNA levels when cultured with osteoblastic cells. MCF-7 cells that overexpress PTHrP, tested in a nude mouse model, caused significantly more bone metastases that was associated with increased osteoclast formation, plasma PTHrP concentrations, and hypercalcemia compared with parental or empty vector controls. A role for PTHrP to decrease Opg expression from osteoblastic cells is supported by two recent publications showing that PTH has this effect on bone cells (Kanzawa et al., 2000; Onyia et al., 2000).

C. Proposed Vicious Cycle Mechanism in Osteolytic Bone Metastases

Based on the above data, a proposed mechanism for osteolytic metastases is illustrated in Figure 1. Tumor cells in bone that secrete PTHrP stimulate osteoclastic bone resorption via osteoblast production of RANK ligand. Bone-derived TGFβ, released as a consequence of osteoclastic bone resorption, stimulates tumor production of PTHrP via TGFβ receptors through the Smad and p38 MAP kinase signaling pathways. This vicious cycle involving PTHrP and TGFβ results in the bone destruction associated with breast cancer and defines specific molecular targets for therapy. As is discussed later, other properties of the tumor cell as well as the bone microenvironment may contribute to this vicious cycle by further increasing tumor PTHrP production or by enhancing osteolysis.

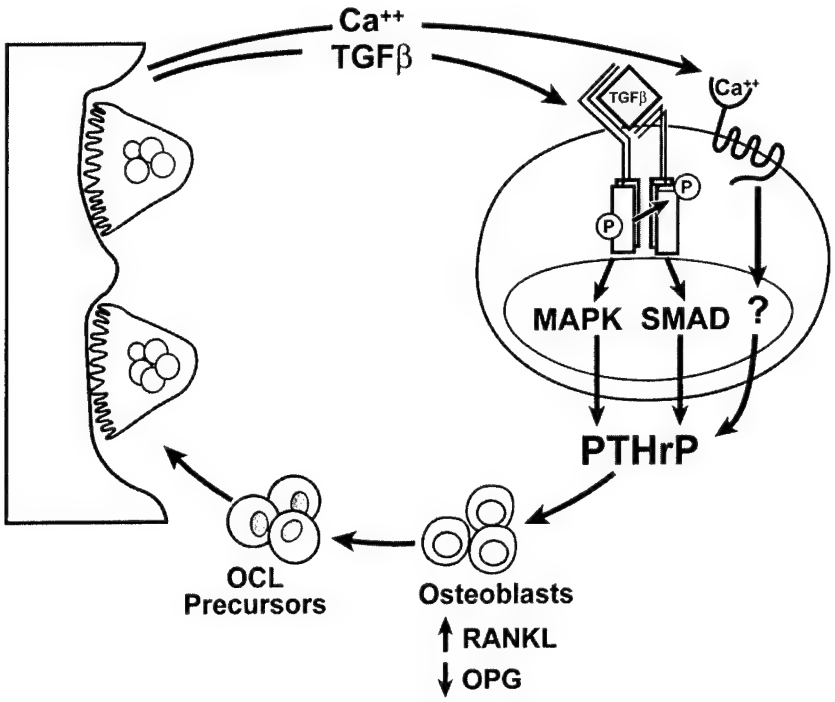

FIGURE 1. Proposed Mechanisms for Osteolytic Bone Metastases: Tumor cells in bone, which secrete PTHrP, stimulate osteoclastic bone resorption via osteoblast production of RANK ligand. The actions of RANKL are opposed by its decoy receptor, osteoprotegerin (OPG). PTHrP also reduces the osteoblast production of OPG to favor osteoclastic bone resorption. Bone-derived TGFβ, released as a consequence of osteoclastic bone resorption, acts on the tumor cells via TGFβ receptors to activate Smad and MAP kinase signaling pathways, which stimulate further tumor production of PTHrP. Ionized calcium, released by osteoclastic bone resorption, can also increase PTHrP production by effects on tumor cells through the calcium-sensing receptor [CaSR]. This vicious cycle involving PTHrP and TGFβ results in the bone destruction associated with breast cancer.

V. INVOLVEMENT OF OSTEOLYTIC FACTORS OTHER THAN PTHrP IN METASTASIS

A variety of factors are now known to stimulate osteoclastic bone resorption through increasing the expression of RANK ligand on the surface of cells in the osteoblastic lineage. Some of these also decrease Opg expression from the same cells (Suda et al., 1999). Several of these are candidates to be produced by tumor cells in bone. Of particular potential are IL-6 and -11, which bind to similar receptors that share the common signaling subunit gp130. These two interleukins are secreted by breast cancer cell lines, such as MDA-MB-231, and may be regulated by TGFβ in a manner similar to the regulation of PTHrP (Selander et al., 2000a,b). Similar to PTHrP, overexpression of IL-11 in MDA-MB-231 breast cancer cells increased osteolytic metastases in the mouse bone metastases model (Selander et al., 2000a). The data obtained with PTHrP-neutralizing antibodies (Guise et al., 1996; Yin et al., 1999a) in mice bearing MDA-MB-231 bone metastases indicate that PTHrP is the primary osteolytic factor responsible for bone destruction. The other factors, such as IL-11, may enhance the end-organ effects of PTHrP, as has already been shown for IL-6 (de la Mata et al., 1995). They could also play a central part in stimulating osteolysis by metastatic cancer cells that are PTHrP-negative. A variety of other tumor-secreted factors are also candidates that contribute to the formation of osteolytic metastases (Peterson et al., 1999).

VI. ROLE OF BONE MATRIX-DERIVED FACTORS IN ADDITION TO TGFβ TO ACTIVATE TUMOR CELLS

The list of factors immobilized in bone matrix reported by Hauschka et al. (1986) and Mohan and Baylink (1991) is almost certainly incomplete. For example, IGF binding proteins are likely to be important components of bone matrix. The known factors, like TGFβ, in addition to being initially laid down by cells of the osteoblastic lineage, also have numerous effects on bone cells. Clear roles for factors such as the IGFs and FGFs 1 and 2 on bone marrow stromal cells, as well as direct and indirect effects on cells of the osteoclastic lineage, have not yet been subjected to experimental tests *in vivo*. An extensive literature describes the growth-stimulatory effects of IGF 1 on breast cancer cells, but it is less clear whether IGFs affect osteoclast function in addition to regulating tumor cell growth. This subject is not readily susceptible to experimental clarification. If bone-derived IGFs, for example, have paracrine effects to stimulate growth of expressing tumor cells, then reagents that neutralize the response of the tumor cells to the factor will decrease tumor burden by direct growth inhibition. Under such circumstances, it will be difficult to quantify factor effects on tumor secretion of osteolytic factors *in vivo*. Similar limitations may apply to other factors released from the bone matrix.

VII. EFFECTS OF EXTRACELLULAR CALCIUM ON TUMOR CELLS

As mentioned above, osteoclastic resorption of bone cannot only release high concentrations of stored growth factors into the bone microenvironment, but also increased concentrations of ionized calcium and phosphate, from the dissolution of the hydoxyapatite of mineralized bone. The calcium-sensing receptor is a G-protein coupled seven transmembrane domain receptor that enables cells expressing it to respond to small variations in the concentration of extracellular calcium (Yamaguchi et al., 2000). Recent reports establish a role of calcium-sensing receptor signaling in regulating tumor secretion of PTHrP (Sanders et al., 2000; Buchs et al., 2000). In both studies, extracellular calcium as well as the polycation neomycin stimulated tumor production of PTHrP by the rat H-500 leydig cells, which cause hypercalcemia in rats. Furthermore, cell-surface expression of the calcium-sensing receptor was detected in the H-500 leydig cells (Sanders et al., 2000). Thus, high ionized calcium concentrations at sites of osteolysis may contribute to the vicious cycle by increasing PTHrP production and thence further osteolysis.

VIII. EFFECTS ON OSTEOLYTIC METASTASES MEDIATED BY LOCAL IMMUNE CELLS

Bone marrow contains the cells of the hematopoietic lineage as well as bone cells. These include T and B lymphocytes, dendritic cells, and monocytes and macrophage. Inflammatory mediators released from these cells can have effects on osteoblastic cells as well as direct effects on the osteoclast lineage (Martin et al., 1998). For instance, activated immune cells can produce IL-1 and -6, which stimulate bone resorption (Reddy and Roodman, 1998). RANK ligand was described as a T-cell factor (Wong et al., 1997a,b) prior to the recognition of its central role in control of osteoclast differentiation. There may be cases in which the vicious cycle between tumor and bone is expanded by the inclusion in the loop of T cells between tumor cells and bone cells. Tumor cells could stimulate osteoclasts by increasing RANK ligand expression from T cells (Horwood et al., 1999). Experimental models of bone metastases have relied on nude mice, whose T-cell deficiency may mask such effects. Several interleukins, in particular IL-4, IL-12, and IL-18 (Gillespie and Horwood, 1998), can inhibit osteoclast formation (Reddy and Roodman, 1998; Martin et al., 1998). IL-18 stimulates interferon-γ production, and interferon-γ inhibits osteoclastic bone resorption. However, recent work suggests that IL-18 acts via GM-CSF to alter the proportion of granulocytic versus monocytic precursors in marrow. This would decrease the percentage of M-CSF-dependent precursors which lead, in the presence of RANK ligand, to osteoclasts.

IX. FACTORS ACTING DIRECTLY ON OSTEOCLASTS

IL-1 and TNFα stimulate bone resorption (Pfeilschifter et al., 1989; Reddy and Roodman, 1998; Uy et al., 1997) but do not act through RANK ligand (Suda et al., 1999). Recent work suggests that, at least *in vitro*, TNFα stimulates osteoclast differentiation from precursors maintained in the presence of M-CSF. The differentiated osteoclasts can then be stimulated to resorb bone by treatment with IL-1 (Kobayashi et al., 2000). These results support a pathway leading to bone resorption that is independent of stromal cells and RANK ligand; however, its importance *in vivo* is unclear. Adding further uncertainty is the possibility, mentioned above, that some osteoclastic-stimulating factors may function differently in mouse and humans (Hofbauer et al., 1999). Nearly all experimental modeling of cancer metastasis to bone has used human tumor cells in nude mice. This system would fail to detect tumor-secreted factors that are highly specific for human versus mouse receptors.

X. EFFECTS OF SEX STEROIDS ON TUMOR CELLS

A. Roles of Sex Steroids in Bone Metastases

The two tumor types that mostly commonly metastasize to bone are those of breast and prostate. Both commonly express PTHrP at the primary site and frequently progress to steroid independence, after initial dependence for growth on sex steroid: estrogen in the case of breast and androgen in the case of prostate. The roles of the sex steroids in the formation of bone metastases are little understood. A complication is added by the high bone turnover state that occurs consequent to sex steroid ablation in both males and females that results in bone loss. This state of high bone turnover may mimic the vicious cycle described above and enhance the initial growth of metastatic cells in bone. Prostate cancer metastatic to bone usually displays an osteoblastic response, characterized by net formation of disordered new bone. However, prostatic bone metastases are usually accompanied by increased circulating markers of bone resorption, suggesting an osteolytic component (Garnero et al., 2000).

Direct transcriptional effects of estrogen on PTHrP mRNA have not been detected, but it seems possible that estradiol could have direct effects on tumor expression of osteolytic factors, such as PTHrP, at metastatic sites.

B. Interactions between Estrogen Receptor (ER) and PTHrP–Potential Role in Bone Metastases

Estrogen is a mitogen for breast cancer cells that express ERα, but the role of this steroid receptor in the pathophysiology of bone metastases is unclear. Women with ER-positive primary tumors are more likely to develop bone metastases (Coleman and Rubens, 1987; Brunn Rasmussen and Kamby, 1989). ERα and PTHrP are coexpressed in primary tumors (Henderson et al., 1999), but there is no clear relationship between ER and PTHrP in breast cancer metastasis to bone. Estradiol can increase uterine PTHrP expression (Thiede et al., 1991) and in several breast cancer lines (Funk and Wei, 1998). Women with ER-positive primary tumors are more likely to develop bone metastases. However, limited clinical data available on ER expression in bone metastases due to breast cancer indicate that 60 to 75% are ER-negative (Brunn Rasmussen and Kamby, 1989). Thus, it appears that bone metastases can be ER-negative in patients with ER-positive primary tumors. Recently, three mutations have been identified in ERα genes from metastatic breast tumors, Ser47Thr, Lys531Glu, and Tyr537Asn, which have provided some insight into a role for ERα in bone metastases (Zhang et al., 1997). The first two mutants had similar activity to wild-type ER, but the Tyr537Asn ER mutant demonstrated a potent, estradiol-independent transcriptional activity. The constitutive activity of Tyr537Asn was unaltered by the ligands estradiol, tamoxifen, or ICI 164,384. Tyr537Asn derived from a bone metastases that was ER-negative by ligand binding

analysis. The mutation is located in exon 8, which encodes the helix 12, carboxy-terminal portion of the hormone-binding domain of the ERα, a potential phosphorylation site implicated in hormone binding, dimerization, and hormone-dependent transcriptional activity. Such a mutation may play an active role in breast cancer metastases to bone. Because it does not bind ligand, tumors expressing it traditionally would be classified as ER-negative. Bone metastases are infrequently sampled, so the prevalence of ERα mutations is unknown. However, the same mutation was independently identified in an endometrial carcinoma (Kohler et al., 1995).

To test the role of ERα in bone metastases, the cDNA for the ERα (Tyr537Asn) mutant was stably expressed in MDA-MB-231 cells (which are ERα-negative). Clonal MDA-MB-231/ERα (Tyr537Asn) lines were transiently transfected with the estrogen response element linked to luciferase (ERE-luc). Compared with empty vector control, these lines showed ER-mediated activity in the absence of 17β-estradiol. Transcriptional activity of the stable clones was not affected by estradiol treatment, but TGFβ1 increased ERE-luc activity. Basal as well as TGFβ-stimulated PTHrP secretion by the Tyr537Asn ER mutant clones was increased compared with the empty vector controls (Yin et al., 1999c). The data suggest that ERα-mediated transcription may increase tumor production of PTHrP. This, plus the ability of TGFβ to enhance ERα-mediated transcription (and possibly growth) may contribute to the propensity of breast cancer to metastasize to the skeleton.

Stable MDA-MB-231 cell lines that expressed wild-type ERα, or ERα mutants that were identified in soft tissue metastases, Ser47Thr and Lys531Glu, were made to determine if the effects of TGFβ on ER-mediated transcription were specific to the ERα (Tyr537Asn) mutant. ER-mediated transcription was increased by17β-estradiol in clones expressing wild-type or Ser47Thr and Lys531Glu mutants. However, there was no additional effect of TGFβ, nor did the combination of 17β-estradiol and TGFβ increase PTHrP production, compared to TGFβ alone. The transcriptional activity of wild-type ERα and the Ser47Thr or Lys531Glu mutants was similar.

The results suggest that the Tyr537Asn substitution induces conformational changes in helix 12 of the ER, mimicking hormone binding and conferring a constitutive transactivation function to the receptor. TGFβ further increases the constitutive ER-mediated transcription. This constitutively active mutant ER, in combination with TGFβ, enhanced tumor cell production of PTHrP. These effects appear to be specific to the Tyr537Asn mutant and suggest cross-talk between the TGFβ signaling pathway and the mutant ERα. Yanagasawa et al. (1999) have found interaction between TGFβ and vitamin D signaling pathways via Smad transcriptional coactivators. The clinical relevance of these observations merits further investigation.

XI. TUMOR-SECRETED FACTORS OPPOSING OSTEOLYSIS

Calcitonin is the only well-characterized endogenous protein factor that directly inhibits osteoclastic bone resorption (Reddy and Roodman, 1998). It binds to receptors expressed on mature osteoclasts. A number of interleukins—4, 12, and 18—and IFγ are discussed in Section VII as inhibitors of osteoclast formation. It was recently shown that systemic infusion of an antibody that neutralized IL-18 decreased osteolytic metastases due to MDA-MB-231 breast cancer cells inoculated into nude mice (Nakata et al., 1999).

About 15% of bone metastases due to breast cancer and a much greater percentage of those due to prostate cancer are predominantly osteoblastic. The metastases are characterized by net bone formation, although markers of bone resorption are often elevated. PTHrP is commonly expressed by the cancer types, but it is unclear whether the osteoblastic response involves antagonism of the osteolytic phenotype. It remains to be tested experimentally whether tumor cells metastatic to bone secrete factors that oppose osteolysis. Such factors could decrease RANK ligand, increase Opg, or have other actions directly on osteoclastic cells or indirectly through immune cells and cells of the osteoblast lineage.

XII. ANGIOGENESIS AND OSTEOLYTIC METASTASES

Bone marrow is a highly vascularized compartment and the site of extensive hematopoiesis. In the remodeling of cortical bone the forming Haversian canal is characterized by osteoclasts resorbing bone immediately followed by the growing blood vessel. Several of the factors stored in bone matrix, such as FGFs 1 and 2, are angiogenic (Keshet and Ben-Sasson, 1999). The intimate associations between bone and blood vessels suggest parallel associations between bone resorptive factors and angiogenic factors (Pluijm et al., 2000; Suda et al., 2000). Such possible relationships are largely unexplored but offer productive avenues for future investigation. A factor of potential interest is platelet-derived endothelial cell growth factor (PD-ECGF; Griffiths and Stratford, 1997). This factor is nonclassically secreted, like IL-1, IL-18, FGFs 1 and 2, and autocrine motility factor (AMF; discussed below). Rather than being targeted to the secretory pathway through the endoplasmic reticulum by a cotranslationally cleaved signal peptide, these proteins are translated and complete folding in the cytosol. They are subsequently released across the plasma membrane by an unknown mechanism. Like AMF, PD-ECGF is a multifunctional, moonlighting protein (Jeffery, 1999). In addition to its extracellular function in angiogenesis, intracellular PC-ECGF is the enzyme thymidine phosphorylase, the target of 5-fluorouracil chemotherapy. Another candidate angiogenic factor is vascular endothelial growth factor (VEGF), that can substitute for M-CSF as the permissive factor that supports the osteoclast lineage (Niida et al., 1999; Nakagawa et al., 2000). It has been reported recently that the C-terminal region of PTHrP can induce VEGF expression in bone cells (Esbrit et al., 2000). Antiangiogenic therapies could have useful applications in the treatment of bone metastases (Wood et al., 2000).

XIII. TUMOR-SECRETED FACTORS ENHANCING INVASIVENESS

Much of the experimental work described in this review in support of the vicious cycle model of osteolytic metastases has used nude mice in which human tumor cells are inoculated via the left cardiac ventricle. Although a murine breast cancer model of spontaneous bone metastases has been described recently (Lelekakis et al., 1999), there are presently no animal models in which syngeneic tumor cells will efficiently and reproducibly metastasize to bone from the site of the primary tumor in such a way to allow rigorous quantitative analysis. The aforementioned spontaneous bone metastases model is limited by the fact that the mice die quickly from metastases to soft tissues, which limits the analysis of bone metastases.

Cancer cell expression of factors affecting motility are important in the general metastatic process, as well as in those processes specific to bone metastasis. The complete metastatic cascade requires tumor cells at the primary site to undergo an extensive series of steps: initial invasiveness through the extracellular matrix of the tumor, intravasation across local vascular endothelium into the blood stream, transport through the circulation to distant sites, extravasation from the blood stream at the site of metastasis, and finally expansion from micro- to macrometastasis (Nicholson, 1988). Migration through extracellular matrices and crossing the vascular wall twice require invasive properties not characteristic of sedentary cells. The migration, however, must be regulated so that generalized invasiveness ceases once the micrometastic site is reached. Thus, invasiveness factors are potentially two-edged swords.

A. Proteolytic Factors Secreted by Tumor Cells

A variety of proteolytic enzymes have been exhaustively studied as the secretory products of tumor cells (Koblinski et al., 2000). Proteinases also play a central role in bone turnover (Delaisse et al., 2000). They include ubiquitous lysosomal enzyme precursors such as procathepsins B, D, H, and L, as well as procathepsin K, which is important to osteoclast function and whose expression is restricted to osteoclasts and breast cancer cells (Littlewood-Evans et al., 1997; Gowen

et al., 1999). Of clearer relevance to tumor invasiveness are the matrix metalloproteinases (MMPs; Lochter and Bissell, 1999). The MMPs form an extensive family of soluble and membrane-bound proteases (Nelson et al., 2000), and there also exist several tissue inhibitors of metalloproteinases, TIMPs. Yoneda et al. (1997) showed that transfection of MDA-MB-231 cells with a TIMP-2 expression DNA reduced osteolytic metastasis to bone. Small molecule inhibitors of MMP activity are now in clinical trials and may be effective in suppressing tumor metastases to secondary sites, including bone.

Urokinase-type plasminogen activator (uPA) is a serine protease similar to tissue-type plasminogen activator (tPA). A major function of uPA is proteolysis during cell migration and tissue remodeling. Although both tPA and uPA have been identified in malignant tissue, uPA appears to have a more prominent role in malignancy. It activates plasminogen to plasmin, which then cleaves extracellular matrix components of laminin, fibronectin, and collagen. The rat prostate PA III tumor line causes new bone formation over the scapula of nude mice. Achbarou et al. (1994) used gene transfer techniques to overexpress uPA in the rat prostate cancer cell line, Mat LyLu, by fivefold compared with the same cells expressing empty vector. A separate Mat LyLu cell line that expressed uPA mRNA in the antisense orientation had a threefold reduction in uPA mRNA compared with the empty vector cells. The uPA-overexpressing, -underexpressing, and parental cell lines were compared in a rat model of bone metastases in which tumor cells inoculated into the left cardiac ventricle of rats cause bone metastasis. Rats inoculated with the uPA-overexpressing cell line developed hind limb paralysis sooner than rats inoculated with empty vector Mat LyLu cells. Similarly, rats inoculated with the uPA antisense-expressing Mat LyLu cells developed hind limb paralysis later than rats inoculated with parental or uPA-overexpressing Mat LyLu (Achbarou et al., 1994). Histologic assessment of the sites of tumor metastasis indicated that more metastatic tumor was present sooner in both skeletal and nonskeletal sites of the rats inoculated with the uPA-overexpressing Mat LyLu cell line, compared with those inoculated with the empty vector or antisense cell line. Bone histology indicated that although both osteolytic and osteoblastic lesions were present in both control and experimental rats, the osteoblastic response was the predominant feature in rats bearing the uPA-overexpressing Mat LyLu cells.

Although prostate-specific antigen (PSA) is a widely used clinical marker for prostatic tumor burden, it is also expressed by breast cancer cells and is a member of the kallikrein serine proteinase family (Watt et al., 1986; Monne et al., 1994). It may have effects on bone by cleaving IGF-binding protein-3 (Fielder et al., 1994), activating latent TGFβ (Killian et al., 1993), and inactivating PTHrP (Cramer et al., 1996).

B. Factors Stimulating Tumor Cell Motility

In addition to being able to digest their way through extracellular matrix components, tumor cells metastasizing from a primary site must also have enhanced motility. Once tumor cells arrive in the bone marrow sinusoids, they must move through the sinusoids to bone. Autocrine motility factor (AMF), thymosin β15, and heat-shock protein 27 (hsp27) have emerged as potential factors controlling cell motility. Thymosin β15 increases cell motility, and when its production was decreased by expression of antisense constructs, as reported by Bao et al. (1996), metastases were prevented in the Dunning rat prostate adenocarcinoma model. Several tumor factors have been described (Stoker and Gherardi, 1991) that have autocrine effects on tumor cell motility, including autotaxin (Nam et al., 2000) and AMF (Silletti and Raz, 1996). The latter is a well-characterized marker of metastatic breast cancer (Bodansky, 1954), and its mRNA is increased by heregulin (Talukder et al., 2000). AMF is the extracellular form of a glycolytic enzyme, phosphoglucose isomerase. It is thus functionally similar to PD-ECGF, in being nonclassically secreted and having distinct intracellular and extracellular functions (Jeffery, 1999). AMF has species-specific effects on bone cells, dose-dependently stimulating RANK ligand mRNA and depressing that of Opg in bone marrow stromal cells (Li et al., 2000).

In vivo, however, it is not an osteolytic factor but stimulates periosteal new bone formation in nude mice.

Changes in intracellular signaling in tumor cells can also alter cell motility and metastasis. Introduction of an hsp27 expression vector into the MDA-MB-231 cell line decreased cell motility *in vitro* and bone metastases *in vivo* (Lemieux et al., 1999).

C. Factors Enhancing Tumor Cell Adhesion to Bone

This review focuses on the attachment of tumor cells to basement membranes and to other cells mediated through cell adhesion molecules such as laminin and E-cadherin. Interactions between tumor cells and the bone microenvironment that result in osteolytic bone destruction, mediated by osteoclasts adjacent to the metastatic tumor cells (Nakai et al., 1992). However, it seems likely that the tumor cells express receptors that specify adhesion to bone (Rusciano and Burger, 1992; Guise and Mundy, 1998). In general the specificity of particular tumors for characteristic tissue sites for distant metastasis is likely to be determined by ligand-receptor interactions between tumor cell and the target site (Roy and Mareel, 1992). Experimental evidence supports the idea that tumor cell surface expression of adhesion molecules affects targeting to bone and the development of bone metastasis (Yoneda, 2000). Bone marrow stromal cells express the vascular cell adhesion molecule-1 (VCAM-1), a ligand for $\alpha_4\beta_1$ integrin. Tumor cells expressing $\alpha_4\beta_1$ integrin may preferentially adhere to bone marrow stromal cells to establish bone metastasis. CHO cells transfected with $\alpha_4\beta_1$ caused bone and lung metastases when inoculated intravenously into nude mice compared with only lung metastases in mice similarly inoculated with untransfected CHO cells. Bone metastases were inhibited by antibodies against $\alpha_4\beta_1$ or VCAM-1. Expression of $\alpha_3\beta_1$, $\alpha_6\beta_1$, or $\alpha_v\beta_1$ did not induce bone metastases (Matsuura et al., 1996). VCAM-1 expression can be regulated by IL-18 (Vidal-Vanaclocha et al., 2000).

Many breast cancer cells express the $\alpha_v\beta_3$ integrin receptor that binds the bone matrix protein, osteopontin, as well as vitronectin (Sung et al., 1999). MDA-MB-231 cells expressing high levels of $\alpha_v\beta_3$ caused fewer bone metastases than cells expressing low amounts (Tondravi et al., 1996). Bone sialoprotein peptides containing RGD sequences have been shown to decrease MDA-MB-231 cell adhesion to extracellular bone matrix *in vitro* (van der Pluijm et al., 1996), and cellular responses to bone sialoprotein can be regulated by the activations state of the $\alpha_v\beta_3$ integrin receptor (Byzova et al., 2000). Tumor cell expression of CD44 may mediate binding to osteopontin in bone (Weber et al., 1996). Increasing E-cadherin expression by transfection of MDA-MB-231 cells decreased metastasis to bone (Mbalaviele et al., 1996, 1998). These data emphasize the complex mechanisms underlying the metastatic process. Specific adhesion to bone by tumor cells may continue in the absence of the formation of bony lesions. In the experiments in which osteolysis was experimentally inhibited, with either neutralizing antibody against PTHrP or by TGFβ signaling blockade (Guise et al., 1996; Yin et al., 1999a), tumor cell foci were still detected in the bone marrow cavities of the affected animals.

XIV. SUMMARY

The cellular and molecular mechanisms responsible for osteolytic metastases are complex and involve bidirectional interactions between tumor cells and bone cells. A central role for TGFβ in the pathogenesis of the osteolytic bone metastases from breast cancer is indicated by several lines of evidence: (1) induction of PTHrP via the Smad and p38 kinase signaling pathway, and (2) potentiation of ERα-mediated transcription induced by a constitutively active ERα. In addition, a variety of tumor-expressed factors, such as IL-6 and -11, could stimulate osteoclastic resorption *in vivo*. Similarly, bone matrix derived factors in addition to TGFβ, such as IGFs 1 and 2, FGFs 1 and 2, and extracellular calcium, could also locally stimulate tumor cells adjacent to sites of bone resorption to alter tumor growth and production of osteolytic factors. Thus, other vicious

cycles between tumor and bone could exist, for which the established TGFβ-PTHrP cycle can serve as a paradigm. A better understanding of the osteolytic mechanisms at the molecular level will result in more effective therapy for this devastating complication of cancer.

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Transcriptional activation of cathepsin D gene expression by growth factors

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ABSTRACT

Insulin-like growth factor-I (IGF-I), transforming growth factor α (TGFα) and epidermal growth factor (EGF) induced cathepsin D gene expression and reporter gene activity in MCF-7 human breast cancer cells transiently transfected with a construct (pCD1) containing a −2576 to −124 cathepsin D gene promoter insert. In contrast, IGF-I, but not TGFα or EGF, induced reporter gene activity in cells cotransfected with wild-type estrogen receptor (ER) expression plasmid and a construct (pCD2) containing estrogen-responsive downstream elements from −208 to −101. Promoter deletion and mutational analysis experiments identified four GC-rich sites and an imperfect palindromic estrogen responsive element required for IGF-I activation of the ER (ligand-independent). Subsequent studies with the mitogen-activated protein kinase (MAPK) inhibitor, PD98059, and a serine[118]-ER mutant confirmed the role of the MAPK pathway for IGF-I activation of the ER in MCF-7 cells. Thus, growth factor activation of ER can mediate transactivation vs ER/Sp1 binding to GC-rich sites and represents a novel pathway for ligand-independent ER action. The divergent pathways for IGF-I and TGFα/EGF activation of the ER observed in MCF-7 cells contrast with previous data indicating that pathways for growth factor activation of the ER are dependent on the gene and/or gene promoter and on cell context.

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INTRODUCTION

Cathepsin D is a proteolytic enzyme that is normally localized in lysosomes and functions in protein catabolism. Cathepsin D and the higher molecular procathepsin D precursor are expressed in estrogen receptor (ER)-positive MCF-7 breast cancer cells; 17β-estradiol (E2) induces secretion of procathepsin D and cathepsin D in this cell line (Morisset *et al.* 1986, Biegel & Safe 1990). The human cathepsin D gene contains nine exons and expresses a single 2·2 kb transcript (Augereau *et al.* 1988, Redecker *et al.* 1991). Cathepsin D expression is under complex hormonal and mitogenic control that is cell- and tissue-specific. Human breast cancer cells express up to 30-fold more cathepsin D mRNA than normal mammary cells (Rochefort 1990). In MCF-7 cells, E2 induces up to a 10-fold increase in cathepsin mRNA levels that is not inhibited by cycloheximide, thus indicating an increased rate of transcription (Cavailles *et al.* 1988). In addition, 8-bromo-cAMP, an inducer of intracellular cAMP levels, also induces cathepsin D mRNA in MCF-7 cells (Chalbos *et al.* 1993).

Cathepsin D is transcribed from five start sites; however, transcriptional activation by estrogens is TATA-dependent (Cavailles *et al.* 1993). Initial studies of the cathepsin D gene promoter demonstrated that E2 responsiveness was primarily associated with the −252 to −124 region of the promoter, and various sequences within this region were protected in a DNase I footprinting experiment (Cavailles *et al.* 1991, 1993, Augereau *et al.* 1994). Deletion analysis of the cathepsin D gene promoter in MCF-7 cells identified three regions at −208 to −161, −145 to −119 and −120 to −101 that were required for ERα activation. The upstream sequence contained a GC-rich Sp1 binding site and an estrogen-responsive element half-site (ERE½) that formed an ER/Sp1

protein–DNA complex in gel mobility shift assays (Krishnan *et al.* 1994, 1995). E2-induced transactivation at the overlapping GC-rich site (−145 to −135) was dependent on ERα/Sp1-DNA interactions that did not require direct ER binding to promoter DNA (Wang *et al.* 1998). The downstream region (−120 to −101) contained an imperfect palindromic ERE (Wang *et al.* 1997) and an adjacent E-box motif (Xing & Archer 1998) that are required for E2 responsiveness.

Cavailles and coworkers (1989) previously reported that growth factors induced cathepsin D gene expression, and this study has confirmed that transforming growth factor α (TGFα), epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I) induced chloramphenicol acetyltransferase (CAT) activity in cells transiently transfected with pCD1 (contains a −2576 to −124 cathepsin D gene promoter insert linked to a CAT reporter gene). Deletion analysis of the cathepsin D gene promoter shows that IGF-I-induced activity is primarily associated with ligand-independent activation of ER and subsequent interaction with E2-responsive elements identified within the E2-responsive proximal region (−208 to −101) of the promoter. In contrast, EGF/TGFα do not induce through activation of these ER-dependent promoter elements, illustrating divergent pathways for IGF-I- and EGF/TGFα-mediated induction of cathepsin D gene expression in breast cancer cells. With the exception of two additional GC-rich upstream sequences in the promoter (−208 to −161), both IGF-I and E2 activate promoter-reporter activity through the same elements, and ligand-independent activation by IGF-I requires phosphorylation of Ser^{118} of the ER through the mitogen-activated (protein) kinase pathway (MAPK).

MATERIALS AND METHODS

Chemicals and biochemicals

Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (DME F-12) without phenol red, phosphate-buffered saline (PBS), acetyl coenzyme A (CoA), E2, 100 × antibiotic/antimycotic solution, IGF-I, TGFα, and EGF were purchased from Sigma Chemical Company (St Louis, MO, USA). Fetal calf serum (FCS) was obtained from Intergen (Purchase, NY, USA). Minimum Essential Medium (MEM) was purchased from Life Technologies (Grand Island, NY, USA). [^{14}C]Chloramphenicol (53 mCi/mmol) was purchased from NEN Research Projects (Boston, MA, USA). Poly d(I-C), restriction enzymes (HindIII and BamHI, etc.) and T4-polynucleotide kinase were purchased from Boehringer Mannheim (Indianapolis, IN, USA). Wild-type human ER (hER) expression plasmid was kindly provided by Dr Ming-Jer Tsai, Baylor College of Medicine (Houston, TX, USA) and the HEGO-Ser^{118} mutant ER (mER-Ser^{118}) was kindly provided by Dr D A Lannigan, University of Virginia (Charlottesville, VA, USA) (Joel *et al.* 1998). Oligonucleotides were synthesized and purchased from either Gene Technologies Laboratory (Texas A & M University, TX, USA), Genosys Biotechnologies, Inc. (Woodlands, TX, USA), or Life Technologies. PD98059 was purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA). Plasmid preparation kits were purchased from Qiagen (Santa Clarita, CA, USA). All other chemicals and biochemicals were the highest quality available from commercial sources.

Table 1 lists the oligonucleotide sequences (sense strand) used in this study to prepare the corresponding plasmids. The numbering is based on +1 as the first nucleotide of translation codon as described (Cavailles *et al.* 1993, Augereau *et al.* 1994). The serum response element (SRE) oligonucleotide is derived from the c-fos protooncogene promoter (−325 to −296) (Treisman 1992).

Plasmids

DNA upstream of exon 1 of the human cathepsin D gene was isolated from a lambda clone containing a 10 kg EcoRI insert, by partial digestion with EagI and digestion with EcoRI. The 3′ EagI site corresponds to CGGCCG 10 bases upstream of the start codon in exon 1. This fragment was subcloned into a plasmid vector with a CAT reporter gene (to give pCD1) from which it was released as a 5′ EcoRI to 3′ HindIII 2·65 kb insert. Other constructs used in this study were obtained from the corresponding double-stranded synthetic oligonucleotides and were prepared by ligation of appropriate double-stranded oligonucleotides into pBL/TATA/CAT vector using HindIII and BamHI restriction enzyme sites, as previously described (Krishnan *et al.* 1994, 1995, Wang *et al.* 1997, 1998). All ligation products were transformed into DH 5α competent *E. coli* cells, plasmids were isolated, and correct clonings were confirmed by restriction enzyme mapping and DNA sequencing using a Sequitherm cycle sequencing kit from Epicentre Technologies (Madison, WI, USA). Constructs pCD3 and pCD3m2 are comparable to the wild-type (ER/Sp1-tk-CAT) and mutant (ER/'Sp1'-tk-CAT) constructs previously described (Krishnan *et al.* 1994). Plasmid preparation for

TABLE 1. Oligonucleotide sequences (sense strand) used in this study to prepare the corresponding plasmids

Plasmid	Oligonucleotide sequence
CD2 (−208 to −101)	5′-*AGCTT*CCCCGCCCC**CCGCCC**GGGCGCTGT**GCGTG**CCCGAGG **TTGCCCC**GCCCAGGCCAGGCCCCGCT**CCGCCCCGCCC**CGCG**CA CGC**CGGCCG**CGCCC**ACG**TGAC**CGGTCCG*G-3*′
CD3 (−208 to −161)	5′-*AGCTT*CC**CCGCCCCCCGCCC**GGGCGCTGTGCGTGCCCGAGG **TTGCCCCGCCC**A*G-3*′
CD3m1 (−208 to −161)	5′-*AGCTT*CC**CCGCCCC**CTGTCCGGGCGCTGTGCGTGCCCGAGGT TGCC**CCGCCC**A*G-3*′
CD3m2 (−208 to −161)	5′-*AGCTT*CCTTTTTTCCTGTCCGGGCGCTGTGCGTGCCCGAGGTT GCC**CCGCC**A*G-3*′
CD3m3 (−208 to −161)	5′-*AGCTT*CCTTTTTTCCTGTCCGGGCGCTGTGCGTGCCCGAGGTT GCCTTTTTTA*G-3*′
CD4 (−145 to −119)	5′-*AGCTT***CCGCCCCGCCC**CGCG**CACGC**CGGCCGC*G-3*′
CD4m (−145 to −119)	5′-*AGCTT*CATTTCATACAAGCG**CACGC**CGGCCGC*G-3*′
CD5 (−120 to −101)	5′-*AGCTTG***CGCCC**ACG**TGACC**GGTCCG*G-3*′
CD5m (−120 to −101)	5′-*AGCTT*GCGCCCACGTAAAAGGTCCG*G-3*′
CD6 (−145 to −101)	5′-*AGCTT***CCGCCCCGCCC**CGCG**CACGC**CGGCCG**CGCCC**ACGTG **ACC**GGTCCG*G-3*′
SRE (−325 to −296)	5′-*AGCTT*ACACAGGATGTCCATATTAGGACATCTGC*G-3*′

The HindIII and BamHI linker sequences are italicized. The bold letters indicate the imperfect palindromic ERE sequence, Sp1 binding site, or dioxin response element (DRE), and their mutated bases are underlined. The numbers in parentheses indicate the positions within the 5′-promoter region of cathepsin D gene. The numbering is based on +1 as the first nucleotide of translation codon as described (Cavailles *et al.* 1993, Augereau *et al.* 1994). The SRE oligonucleotide is derived from the c-fos protooncogene promoter (−325 to −296) (Treisman 1992).

transfection utilized alkaline lysis followed by two cesium chloride gradient centrifugations or the Qiagen Plasmid Mega Kit.

Cell culture maintenance and growth

MCF-7 and MDA-MB-231 human breast cancer cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA), and MCF-7 cells were maintained in MEM with phenol red and supplemented with 10% FCS plus 0·2 × antibiotic/antimycotic solution, 0·035% sodium bicarbonate, 0·011% sodium pyruvate, 0·1% glucose, 0·238% Hepes, and 6×10^{-7}% insulin. MDA-MB-231 cells were maintained in DME F-12 media supplemented with 5% FCS, 0·5 × antibiotic/antimycotic solution and 0·22% sodium bicarbonate. Cells were passaged every three to five days without becoming confluent.

Transient transfection and CAT assays

Cells grown under maintenance were trypsinized, seeded in 100-mm petri dishes with 10 ml phenol red-free DME F-12 medium plus 1% charcoal-stripped FCS, and grown until 50–60% confluent. One to three hours prior to transfection, the medium was replaced with 4 ml of the charcoal-stripped DME F-12 medium. Cells were transfected with 1 ml transfection cocktail containing 8 μg test plasmid, 1·5 μg of a β-galactosidase-lacZ plasmid (Invitrogen, Carlsbad, CA, USA), 1·5 μg hER or mER-Ser^{118}, 50 μl 2·5 M $CaCl_2$ and 500 μl 2 × Hepes buffer saline (HBS) (pH 7·05). Due to overexpression of the constructs, cotransfection with ER is required for ER action, and this has previously been reported in MCF-7 cells for other constructs including those that have single palindromic ERE inserts derived from the frog vitellogenin A2 gene promoter and other plasmids containing promoter inserts from E2-responsive cathepsin D, c-myc, retinoic acid receptor α1, progesterone receptor, Hsp 27, c-fos and pS2 genes (Weisz & Rosales 1990, Cavailles *et al.* 1991, Savouret *et al.* 1991, Dubik & Shiu 1992, Cavailles *et al.* 1993, Augereau *et al.* 1994, Krishnan *et al.* 1994, Zacharewski *et al.* 1994, Rishi *et al.* 1995, Porter *et al.* 1996, Sathya *et al.* 1997). After incubation for 14–16 h at 37 °C in air:CO_2 (95%:5%), cells were washed once with 5 ml PBS and treated with growth

factors and/or PD98059. After 24 to 30 h, cells were harvested by scraping. Cells were lysed in 200 µl 0·25 M Tris–Cl (pH 7·6) and freeze-thawed (3 ×) in liquid nitrogen for 2 min. The cell debris was pelleted and the protein concentrations were measured by the method of Bradford (1976) using bovine serum albumin as standard. An aliquot of cell lysate was brought to 120 µl with 0·25 M Tris–Cl (pH 7·6) and incubated with 1 µl [^{14}C]chloramphenicol (53 mCi/mmol) and 40 µl 4 mM acetyl CoA for 4 h at 37 °C. After vortexing with 700 µl ethyl acetate for 30 s, the mixture was centrifuged at 16 000 ***g*** for 1 min at room temperature. A 600 µl aliquot of ethyl acetate was evaporated *in vacuo*, resuspended in 20 µl ethyl acetate, and separated by thin-layer chromatography using a 95:5 chloroform: methanol solvent as previously described (Wang *et al.* 1997, 1998). The percentage protein conversion into acetylated chloramphenicol was quantitated using the counts/min obtained from the Betagen Betascope 603 blot analyzer (Intelligenetics, Mountain View, CA, USA). CAT activity was normalized to β-galactosidase enzyme activity obtained after cotransfection with a β-galactosidase-lacZ plasmid (1·50 µg). Initial studies (see Figs 1, 2, 3A,B) standardized transfections using protein concentrations, whereas results in Figs 3C, 4 and 5 standardized transfections using the cotransfection assay, and both assays gave similar results.

Northern blot analysis

Cathepsin D mRNA levels were determined by using a 1·2-kb EcoRI fragment of the human cathepsin D cDNA. β-Tubulin mRNA levels were determined by using a 1·1-kb EcoRI fragment of human β-tubulin cDNA. Total RNA was isolated by the RNAzol B reagent (TelTest, Friendswood, TX, USA). Total RNA (20 µg) was separated in a 1·2% agarose/1 M formaldehyde gel in 20 mM sodium phosphate/2 mM EDTA, transferred onto a nylon membrane by capillary action, and bound to the membrane by UV crosslinking. cDNAs were labeled with [α-^{32}P]dCTP by using a random primer DNA labeling system (Boehringer Mannheim), and the radiolabeled cDNA probe was added at 2×10^6 c.p.m./ml hybridization solution (5 × SSPE (1 × SSPE is 0·15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7·4), 1% sodium dodecyl sulfate (SDS), 10% dextran sulfate, 5 × Denhardt's solution). Hybridizations were performed in roller bottles at 65 °C for 20 h. Nonspecifically-bound probe was removed by 15 min (2 ×) and 20 min (1 ×) washes at 20 °C in 1 × SSPE, two 30-min washes at 65 °C in 0·1 × SSPE-1% SDS, and one 15-min wash at 20 °C in 1 × SSPE. Membranes were stripped of probe by boiling for 20 min in 0·1 × SSPE-0·5% SDS. Bands were scanned with Adobe Photoshop 3·0 (Mountain View, CA, USA) and quantitated with ZERO-Dscan (Scanalytics, Billerica, MA, USA).

Statistics

Results are expressed as means ± S.E. for at least three independent (replicate) experiments for each treatment group. Statistical significance was determined by ANOVA and Student's *t*-test and the levels of probability are noted for each experiment.

RESULTS

Previous studies have demonstrated that growth factors induce cathepsin D gene expression in MCF-7 cells (Cavailles *et al.* 1989), and the results in Fig. 1 demonstrate that IGF-I significantly induces cathepsin D mRNA levels and also causes a 2·1- to 3·4-fold increase in CAT activity in MCF-7 cells transiently transfected with pCD1 containing a −2576 to −124 cathepsin D gene promoter insert. In addition, both EGF and TGFα also induce CAT activity in MCF-7 cells transfected with pCD1. In contrast, only IGF-I significantly induced CAT activity (2·9-fold) in cells transfected with pCD2 (Fig. 1C) suggesting that the −208 to −101 region of the cathepsin D gene promoter was associated with IGF-I-responsiveness, whereas upstream elements are required for TGFα/EGF action. MCF-7 cells express the IGF-I and EGF receptors (Davidson *et al.* 1987, Cullen *et al.* 1990); however, it is possible that differences between IGF-I and EGF may be due to decreased kinase activation by EGF. Results in Fig. 1D show that IGF-I and EGF activate the pSRE construct indicating that both growth factors induce kinase-dependent activation pathways in MCF-7 cells. Interestingly, IGF-I and E2 activation of constructs derived from the −208 to −101 region of the promoter all required cotransfection with ERα expression plasmid, whereas growth factor activation of pSRE did not require further expression of growth factor receptors.

Three E2-responsive regions of the cathepsin D gene promoter have been characterized at −208 to −161, −145 to −119, and −120 to −101 (Krishnan *et al.* 1994, 1995, Wang *et al.* 1997, 1998, Xing & Archer 1998), and these sequences have been inserted upstream from a CAT gene sequence in pBL/TATA/CAT2 to give pCD3, pCD4 and pCD5. Results illustrated in Fig. 2 show that IGF-I, but not TGFα or EGF, induced CAT activity (3·3-fold induction for IGF-I) in cells

transiently transfected with pCD3. Similar results were obtained with MCF-7 cells transfected with pCD4 and pCD5 in which treatment with IGF-I induced a 2·0- and 2·6-fold increase in CAT activity, respectively, whereas induction responses were not observed for EGF and TGFα. Induction responses by IGI-I required cotransfection with wild-type ER expression plasmid (data not shown).

Previous studies have demonstrated that elements required for ERα-dependent activation of pCD3, pCD4 and pCD5 include the GC-rich sites in the former two constructs and an imperfect palindromic ERE overlapping an E-box respectively (Krishnan *et al.* 1994, 1995, Wang *et al.* 1997, 1998, Xing & Archer 1998). Therefore, the role of these sites in IGF-I-mediated transactivation was investigated using mutant constructs pCD3m, pCD4m and pCD5m that are not E2-responsive. The results (Fig. 3) clearly demonstrate that in cells transiently transfected with the mutant pCD4m and pCD5m constructs, IGF-I did not induce reporter gene expression, whereas induction was observed using the corresponding wild-type constructs. The −208 to −161 region of the cathepsin D gene promoter contains an E2-responsive $Sp1(N)_{23}ERE½$ motif and mutation of either the Sp1 binding site or ERE½ results in loss of E2 responsiveness (Krishnan *et al.* 1994, 1995). However, the results in Fig. 3C show that IGF-I induced CAT activity using the pCD3m1 construct mutated in the GC-rich site that forms part of the $Sp1(N)_{23}ERE½$ motif. The −208 to −161 region also contains two additional GC-rich sites at −206 to −201 and −167 to −162, and pCD3m2 and pCD3m3 contain

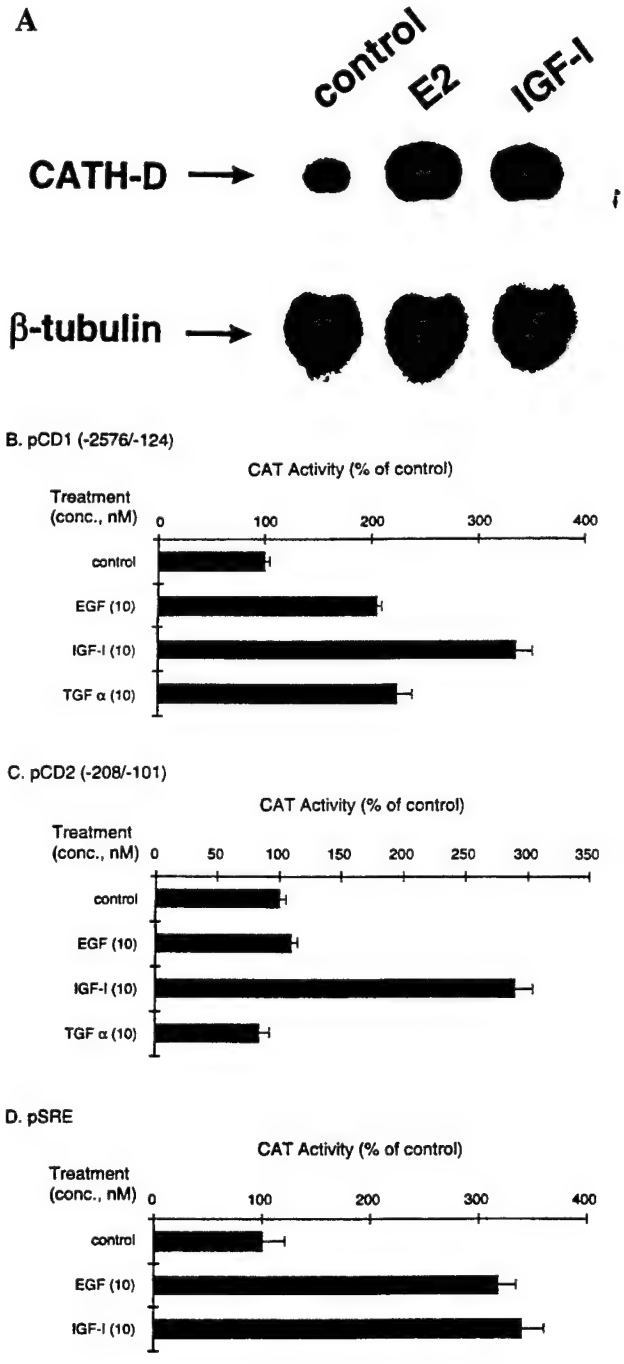

FIGURE 1. Growth factor-mediated induction of cathepsin D. (A) E2- and IGF-I-mediated cathepsin D (CATH-D) gene expression. Total RNA was isolated from MCF-7 cells treated with dimethyl sulfoxide (DMSO) (control), 10 nM E2, or 10 nM IGF-I for 24 h. The Northern blot was visualized by autoradiography and quantitated as described in Materials and Methods. Cathepsin D mRNA levels were normalized to β-tubulin mRNA for each treatment group, and relative cathepsin D mRNA levels were 100 ± 19 (control), 264 ± 12 (E2), and 232 ± 6 (IGF-I). There was a significant increase ($P<0·05$) in cathepsin D mRNA levels in E2- or IGF-I-treated cells. (B) Transfection with pCD1. MCF-7 cells were cotransfected with pCD1 and wild-type ER, treated with DMSO, 10 nM IGF-I, EGF or TGFα, and CAT activity was determined as described in Materials and Methods. Significant induction ($P<0·05$) was observed for all three growth factors. (C) Transfection with pCD2. This experiment with pCD2 was carried out as described in (B). Significant induction ($P<0·05$) was observed only for IGF-I. (D) IGF-I and EGF induction with pSRE. MCF-7 cells were transfected with pSRE (no ER expression plasmid), treated with 10 nM IGF-I or EGF, and CAT activity determined as described in (B). Both growth factors significantly induced ($P<0·05$) CAT activity. Results are presented as means ± S.E. for three replicate experiments for each treatment group.

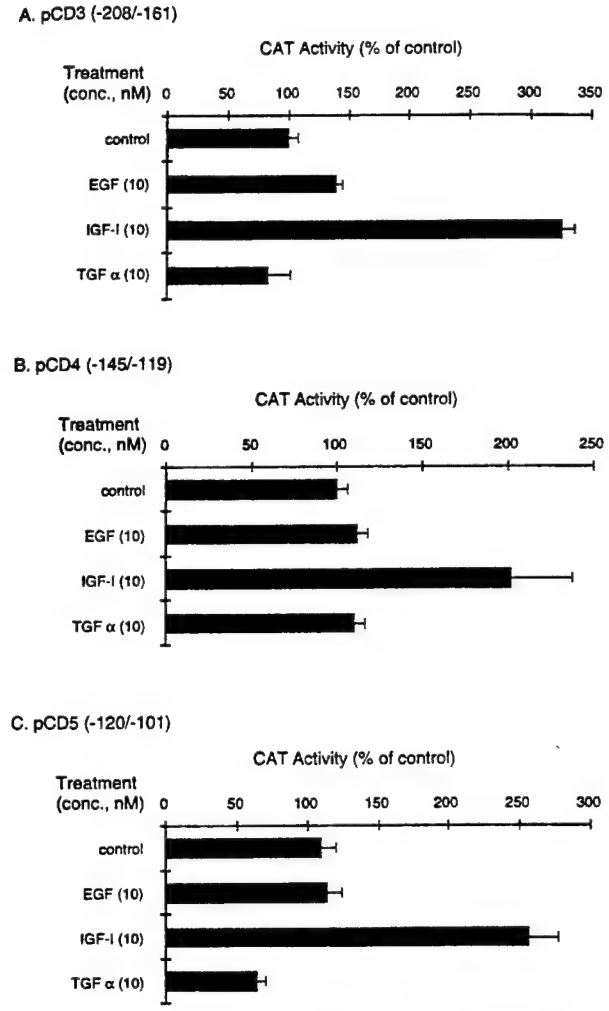

FIGURE 2. Growth factor induction after transfection with pCD3, pCD4 and pCD5. MCF-7 cells were transfected with pCD3 (A), pCD4 (B) or pCD5 (C), and wild-type ER expression plasmid and treated with 10 nM IGF-I, EGF or TGFα. CAT activity was determined as described in Materials and Methods. IGF-I significantly ($P<0{\cdot}05$) induced CAT activity with all constructs, whereas EGF and TGFα were inactive. Results are presented as means ± S.E. for three replicate experiments for each treatment group.

a second mutation and mutations at all three GC-rich sites respectively. The results show that IGF-I induces reporter gene activity in MCF-7 cells transfected with pCD3m2, whereas no induction response is observed with pCD3m3. Thus, all three GC-rich sites can play a role in ligand-independent ER activation by IGF-I of pCD3, and this represents a new pathway for growth factor-mediated transactivation through an ER/Sp1 complex.

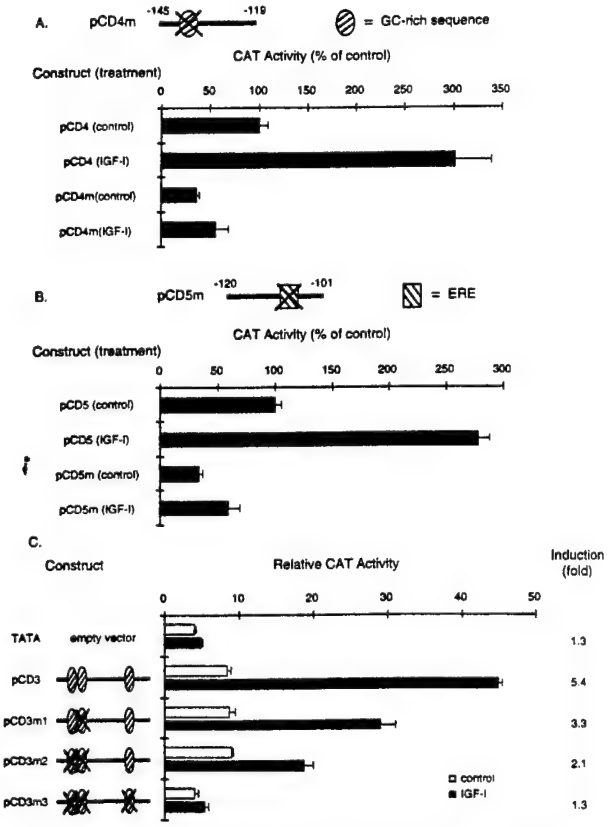

FIGURE 3. Induction of mutant constructs by IGF-I. (A) pCD4m. Wild-type pCD4, pCD4m, and ER expression plasmid were transfected into MCF-7 cells, and CAT activity was determined in the various treatment groups as described in Fig. 1. IGF-I induced CAT activity only in cells transfected with wild-type pCD4, but not pCD4m. (B) pCD5m. Wild-type pCD5 and mutant pCD5m were transfected as described above, and IGF-I induced CAT activity only with wild-type pCD5 and not pCD5m. (C) Variant pCD3 constructs. MCF-7 cells were transfected with wild-type pCD3 or one of three mutants (pCD3m1, pCD3m2 or pCD3m3), and IGF-I responsiveness was determined as described above. CAT activity was significantly induced ($P<0{\cdot}05$) in cells transfected with wild-type pCD3 or mutant pCD3m1 or pCD3m2 constructs; however, after mutation of all three GC-rich sites (pCD3m3), CAT activity was not induced by IGF-I. Only mutations of the GC-rich site in the $Sp1(N)_{23}ERE½$ motif (Krishnan *et al.* 1994) resulted in loss of E2-responsiveness with these constructs (data not shown). Results are presented as means ± S.E. for three replicate experiments for each treatment group.

It has been reported that IGF-I-induced activation of ER was dependent on the MAPK pathway (Kato *et al.* 1995); therefore, the effects of the MAPK inhibitor PD98059 were determined in MCF-7 cells transiently transfected with pCD3,

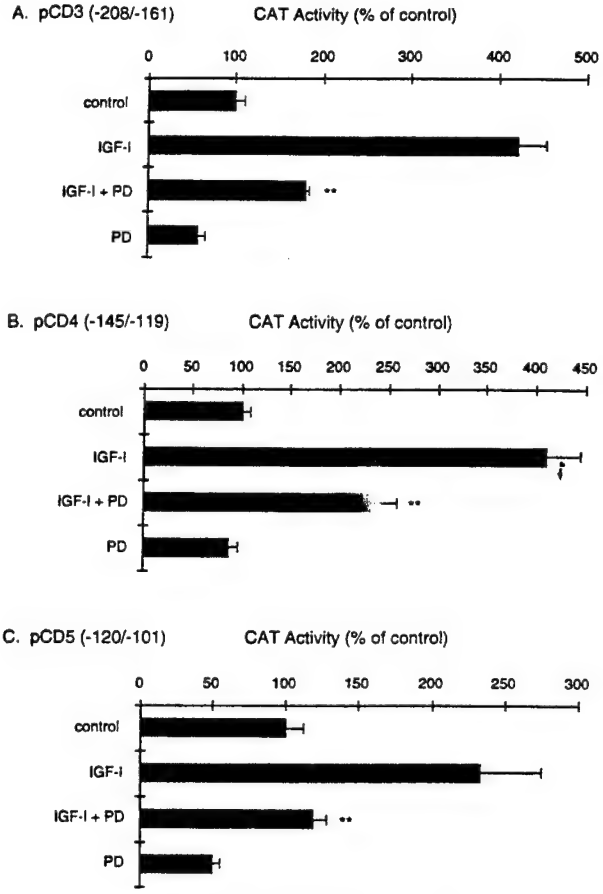

FIGURE 4. Effects of PD98059 (PD) on IGF-I activation of (A) pCD3, (B) pCD4 and (C) pCD5. Transfection experiments were carried out as described in Materials and Methods, and the effects of PD98059 alone or in combination with 10 nM IGF-I were also determined. PD98059 significantly inhibited (**$P<0{\cdot}05$) IGF-I-induced CAT activity in MCF-7 cells transfected with pCD3, pCD4 and pCD5. Results are presented as means ± S.E. for three replicate experiments for each treatment group.

pCD4 or pCD5 and treated with IGF-I (Fig. 4). The results show that IGF-I-induced CAT activity is significantly inhibited by PD98059 in MCF-7 cells transfected with all three constructs, suggesting a role for the MAPK pathway in IGF-I-mediated ER activation of cathepsin D.

Previous studies have demonstrated that MAPK-dependent phosphorylation of Ser^{118} plays an important role in growth factor activation of ER (Kato *et al.* 1995); therefore, we investigated the role of Ser^{118} using a mutant ER-Ser^{118} expression plasmid in ER-negative MDA-MB-231 breast cancer cells transfected with E2-responsive constructs derived from the cathepsin D gene promoter. Preliminary studies showed that pCD4 and pCD5 were only weakly inducible by IGF-I in MDA-MB-231 cells, and therefore a combined construct (pCD6), previously shown to be E2-responsive (Wang *et al.* 1997), was utilized for this study. IGF-I induced reporter gene activity in MDA-MBA-231 cells transiently cotransfected with pCD3 or pCD6 and wild-type ER, whereas no induction was observed when cells were cotransfected with mutant ER-SER^{118} expression plasmid (Fig. 5). The comparative activation of pCD3 in MCF-7 cells transfected with wild-type ER and ER-Ser^{118} gave similar results except that some induction was observed, and this may be due to endogenous expression of wild-type ER in this cell line. These results suggest the important role of Ser^{118} phosphorylation of ER by IGF-I for activation of cathepsin D by ER (ligand-independent) in breast cancer cells.

DISCUSSION

Growth factors such as EGF, TGFα, IGF-I and insulin induce proliferation of ER-positive breast cancer cells and modulate expression of several E2-responsive genes (Furlanetto & DiCarlo 1984, Bates *et al.* 1988, Dickson & Lippman 1988, Karey & Sirbasku 1988, Wilding *et al.* 1988, Cavailles *et al.* 1989, Freiss *et al.* 1990, Katzenellenbogen & Norman 1990, Dickson & Lippman 1991, Fernandez *et al.* 1994, Westley & May 1994, Dickson & Lippman 1995, Smith 1998). EGF also exhibits estrogen-like activity in the mouse female genital tract (Nelson *et al.* 1991), and *in vitro* studies in endometrial and other ER-negative cell lines show that EGF induces reporter gene activity in cells transiently transfected with constructs containing a perfect palindromic ERE promoter (Ignar-Trowbridge *et al.* 1992, 1993, 1996, Kato *et al.* 1995, El-Tanani & Green 1997). IGF-I also exhibits some of the same responses both *in vivo* and *in vitro* (Murphy & Ghahary 1990, Aronica & Katzenellenbogen 1993, Ignar-Trowbridge *et al.* 1996), and both growth factor-mediated responses are dependent on activation function 1 (AF-1) of the ER. Kato and coworkers (1995) also showed that AF-1-dependent transactivation in ER-negative COS and HeLa cells is modulated through ras-MAPK-mediated phosphorylation of ER-Ser^{118}.

Previous studies have reported that IGF-I and EGF induce cathepsin D gene expression in ER-positive breast cancer cells (Cavailles *et al.* 1989); this was also noted in this study and comparable induction of CAT activity was observed in MCF-7 cells transiently transfected with pCD1

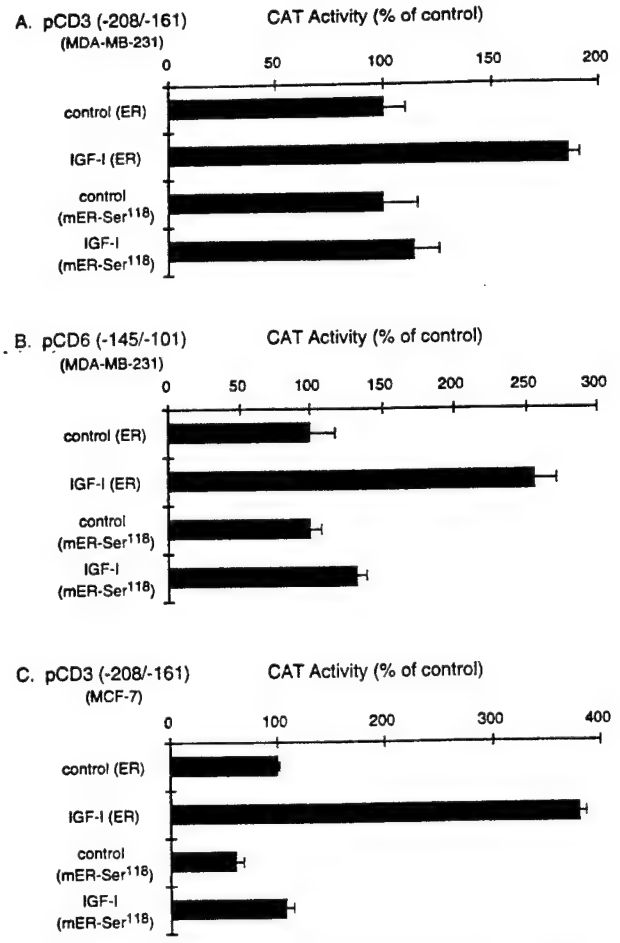

FIGURE 5. Role of Ser^{118} in activation of ER by IGF-I. ER-negative MDA-MB-231 cells were transiently cotransfected with (A) pCD3 or (B) pCD6, wild-type ER, or mutant mER-Ser^{118}, and (C) MCF-7 cells were transfected with pCD3, wild-type ER or mutant mER-Ser^{118}, treated with DMSO or IGF-I, and CAT activity was determined as described in Materials and Methods. IGF-I significantly ($P<0{\cdot}05$) induced CAT activity in MDA-MB-231 and MCF-7 cells cotransfected with wild-type ER, whereas decreased (MCF-7 cells) or no significant induction (MDA-MB-231 cells) was observed in cells transfected with mutant ER-Ser^{118}. Results are presented as means ± S.E. for three replicate experiments for each treatment group.

and a construct containing a serum response element promoter (pSRE) (Fig. 1). Thus, elements required for responsiveness to IGF-I, EGF and TGFα are present in the −2576 to −124 region of the cathepsin D gene promoter, and both IGF-I and EGF activate through a serum response element indicating that functional growth factor receptors are expressed in these cells, as previously described (Furlanetto & DiCarlo 1984, Davidson

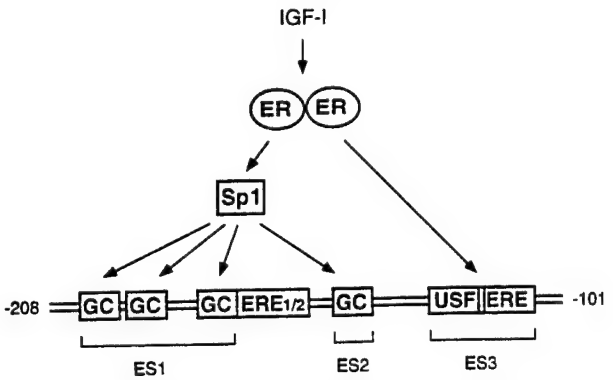

FIGURE 6. Model for IGF-I-ER activation of cathepsin D gene promoter elements, ES1, ES2 and ES3.

et al. 1987, Cullen *et al.* 1990, Freiss *et al.* 1990). E2-responsiveness of the cathepsin D gene is primarily located in the downstream proximal region of the promoter (Cavailles *et al.* 1991, Augereau *et al.* 1994), and at least three E2-responsive motifs have been identified (Krishnan *et al.* 1994, 1995, Wang *et al.* 1997, 1998, Xing & Archer 1998). The role of the ER in mediating growth factor signaling was investigated using both wild-type and mutant constructs containing cathepsin D gene promoter elements derived from the downstream E2-responsive motifs, ES-1, ES-2 and ES-3 (Fig. 6). Results (see Figs 1 and 2) clearly show that IGF-I, but not EGF or TGFα, activates gene expression through these E2-responsive elements, and this distinctly differentiates between IGF-I and EGF/TGFα activation of the cathepsin D gene. Similar differences have also been observed in MCF-7 cells transfected with a construct containing a perfect palindromic ERE promoter; IGF-I and E2 induced reporter gene activity (Hafner *et al.* 1996, Lee *et al.* 1997), whereas EGF was inactive (Hafner *et al.* 1996). Previous studies reported a common pathway for growth factor-ER signaling in ER-negative cells and in ER-positive BG-1 ovarian adenocarcinoma cells (Nelson *et al.* 1991, Ignar-Trowbridge *et al.* 1993, 1996, Kato *et al.* 1995, El-Tanani & Green 1997); however, results of this study show divergent pathways for IGF-I-ER and TGFα/EGF-ER action, demonstrating that pathways for growth factor activation of the ER are cell context- and possibly promoter-dependent. Current studies are focused on characterizing upstream elements in the cathepsin D gene promoter associated with EGF/TGFα-responsiveness in breast cancer cells.

Confirmation of IGF-I action on E2-responsive motifs within the −201 to −101 region of the cathepsin D gene promoter was determined using

mutant constructs (Fig. 3), the MAPK inhibitor PD98059 (Fig. 4), and mER-Ser^{118} (Fig. 5). The results were consistent with IGF-I activation of ER through the ras-MAPK pathway and confirmed that phosphorylation of SER^{118} was important from ligand-independent ER activation by IGF-I. One surprising result was obtained with mutants derived from the −208 to −161 region of the cathepsin D gene promoter. Mutation of the GC-rich site (−199 to −194) that forms part of the Sp1(N)$_{23}$ERE½ motif, to give pCD3m1 (Fig. 3C) did not result in loss of IGF-I responsiveness, whereas this construct is not E2-responsive in MCF-7 cells (Krishnan *et al.* 1994). However, this region of the promoter contains two additional GC-rich sites and IGF-I mediated transactivation of mutant pCD3 constructs is not lost until all three Sp1 binding sites are mutated. Thus, ligand-independent activation of ER by IGF-I may involve the three GC-rich sites in pCD3, whereas ligand-dependent activation of pCD3 by E2 is more selective and is activated through only one of these sites (Krishnan *et al.* 1994).

These studies demonstrate that the pattern of growth factor activation of the ER is complex and is dependent on the gene promoter and cell context. Previous studies indicate that TGFα, EGF and IGF-I induce cell proliferation and gene expression in MCF-7 cells (Furlanetto & DiCarlo 1984, Bates *et al.* 1988, Dickson & Lippman 1988, Karey & Sirbasku 1988, Wilding *et al.* 1988, Cavailles *et al.* 1989, Freiss *et al.* 1990, Katzenellenbogen & Norman 1990, Dickson & Lippman 1991, Fernandez *et al.* 1994, Westley & May 1994, Dickson & Lippman 1995, Smith 1998); however, results of this study demonstrate that only IGF-I activates ER (ligand-independent)-mediated induction of reporter gene activity through multiple E2-responsive elements in the proximal promoter region of the cathepsin D gene. These data indicate divergent pathways for IGF-I and TGFα/EGF in breast cancer cells. Current studies in this laboratory are focused on identifying specific factors responsible for cell-specific differences in growth factor activation of the ER.

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The Crystal Structure of Human Phosphoglucose Isomerase at 1.6 Å Resolution: Implications for Catalytic Mechanism, Cytokine Activity and Haemolytic Anaemia

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Phosphoglucose isomerase (PGI) is a multifunctional protein, which, inside the cell, functions as a housekeeping enzyme of glycolysis and gluconeogenesis and, outside the cell, exerts wholly unrelated cytokine properties. We have determined the structure of human PGI to a resolution of 1.6 Å using X-ray crystallography. The structure is highly similar to other PGIs, especially the architecture of the active site. Fortuitous binding of a sulphate molecule from the crystallisation solution has facilitated an accurate description of the substrate phosphate-binding site. Comparison with both native and inhibitor-bound rabbit PGI structures shows that two loops move closer to the active site upon binding inhibitor. Interestingly, the human structure most closely resembles the inhibitor-bound structure, suggesting that binding of the phosphate moiety of the substrate may trigger this conformational change. We suggest a new mechanism for catalysis that uses Glu357 as the base catalyst for the isomerase reaction rather than His388 as proposed previously. The human PGI structure has also provided a detailed framework with which to map mutations associated with non-spherocytic haemolytic anaemia.

Keywords: aldose-ketose isomerases; neuroleukin; cytokine; haemolytic anaemia; X-ray crystallography

Introduction

Glucose 6-phosphate isomerase (E.C. 5.3.1.9) (also known as phosphoglucose isomerase (PGI) and hexose phosphose isomerase), hereinafter referred to as PGI, has traditionally been considered a rather pedestrian enzyme of glycolysis and gluconeogenesis. In these pathways PGI interconverts glucose 6-phosphate (G6P) and fructose 6-phosphate (F6P), a reaction driven solely by the relative proportions of these sugars in the cytosol. The reaction proceeds *via* an acid-base mechanism involving a *cis*-enediol intermediate.[1] Kinetic data have suggested the participation of ionisable groups with pK_a values of ~6.5 and ~9.5 in the catalytic mechanism. In one proposed mechanism these have been interpreted as being due to histidine and lysine residues acting as general acid-base catalysts in proton abstraction and sugar ring-opening, respectively.[2] Two recent crystal structures of PGI from *Bacillus*,[3] and rabbit muscle[4] show that candidate histidine and lysine residues are present at the active site.

The view of PGI as a simple housekeeping enzyme changed following a series of discoveries linking PGI to a variety of cytokine activities. In these guises PGI has been rediscovered as neuroleukin (NLK),[5] autocrine motility factor (AMF),[6] maturation factor (MF),[7] and, more recently, myofibril-bound serine proteinase inhibitor (MBSPI).[8] It now appears to act in a bewildering array of extracellular processes.[9] Thus, PGI is a product of T

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Abbreviations used: PGI, phosphoglucose isomerase; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; NLK, neuroleukin; AMF, autocrine motility factor; MF, maturation factor; MBSPI, myofibril-bound serine proteinase inhibitor; PK, pyruvate kinase; TPI, triose phosphate isomerase.

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cells that promotes the survival of spinal and sensory neurons *in vitro* and stimulates the production of immunoglobulin by B cells.[10] It mediates the differentiation of human myeloid leukaemia cells[7] and is a tumour cell product that promotes cell migration.[6] The latter may be related to the high concentrations of PGI observed in the sera of patients with certain cancers.[11] Finally, PGI is the antigen in a mouse model of autoimmune rheumatoid arthritis[12] and is a major surface antigen in sperm agglutination.[13]

How PGI acts in these varied systems remains a mystery, and is the subject of intense investigation. There are several apparent contradictions that remain to be resolved. The extracellular cytokine activities have been associated with a monomer of molecular mass of ~55kD.[5,8,14,15] This is contrary to the established dimeric structure of PGI, comprising identical subunits, each of molecular mass 63 kDa. In fact, a dimer of PGI is prerequisite for catalytic activity[16] and this is consistent with the active site of the enzyme being composed of polypeptide chains from both subunits[3,4] (C.D. & H.M., unpublished results). Yet, at least in the case AMF, active site inhibitors of PGI also inhibit its cytokine activity.[6,17] This apparent structural overlap between the catalytic and cytokine activities of PGI precludes the simplistic notion that the cytokine form of PGI is a truncated monomer, which is functionally distinct from the dimeric enzymatic form of PGI.

PGI is additionally important because deficiency in this enzyme leads to non-spherocytic haemolytic anaemia,[18] an autosomal recessive genetic disorder caused by defects in pyruvate kinase (PK), triose phosphate isomerase (TPI) or PGI. Many of the PGI variants have now been characterised at the molecular level (for a review, see ref. 19). In the absence of detailed structural information for human PGI, however, the effect of these mutations on the phenotype cannot be assessed directly.

Structural investigations of PGI have been pursued for many years,[20–22] but only recently has high resolution structural information for this enzyme become available from *Bacillus*,[3] rabbit[4] (C.D. & H.M., unpublished results) and pig (C.D. & H.M., unpublished results). Here, we present the crystal structure of PGI from human, solved at 1.6 Å. At this resolution the active site is revealed in considerable detail. A sulphate ion from the crystallisation solution has bound to the active site where it mimics the binding of phosphate of the sugar substrates. In addition the structure provides a framework for the interpretation of mutations giving rise to haemolytic anaemia.

Results

Structure determination

Crystals were obtained over wells containing 2.2-2.6 M ammonium sulphate buffered with Tris-HCl (pH 8.0-8.5) or bis-Tris propane (pH 9.0). The best crystals, and those used for the structural studies, grew over wells containing 2.6 M ammonium sulphate, 100 mM Tris (pH 8.5). These were of approximate dimensions 0.5 mm × 0.5 mm × 0.4 mm and had a multi-faceted, diamond-like morphology. The crystals belong to space group $P4_32_12$ with cell dimensions $a = b = 94.18$ Å and $c = 136.14$ Å. The diffraction of these crystals was excellent: the data collection statistics are shown in Table 1. Based on the estimate of solvent content,[23] there is a single PGI monomer in the asymmetric unit. Interestingly, these crystals are essentially isomorphous with PGI crystallised from pig muscle,[20] which reflects the high degree of sequence identity between the two enzymes (93%). The structure was therefore solved relatively easily by simple refinement of the 2.5 Å structure of PGI from pig muscle (C.D. & H.M., unpublished results). Continuous electron density was observed for all of the main chain residues (Ala1 through to Glu557). Two peaks of significant density were observed in the active site that could not be interpreted as water molecules. One of these had a tetrahedral shape and was assigned to a sulphate molecule. The other was extended with no visible branching and was interpreted as molecule of β-mercaptoethanol. The final refinement statistics are shown in Table 1 and the final electron density in the active site region is shown in Figure 1.

Structure description

The structure of human PGI is highly similar to that of the rabbit enzyme[4] (C.D. & H.M., unpublished data) and so will be described only briefly. The enzyme is comprised of two domains, traditionally termed large and small,[21] although they are fairly similar in size (Figure 2). Each domain is

Table 1. X-ray diffraction data and refinement statistics

Resolution range (Å)	30-1.62 (1.69-1.62)
R_{merge} (%)	4.8 (17.3)
Redundancy	5.5 (5.5)
Completeness	96.4 (88.4)
$\langle I \rangle / \langle \sigma I \rangle$	30.7 (7.3)
No. unique reflections	74,713
No. reflections used in refinement	70,947
No. reflections in free R set	3766
No. of protein atoms	4440
No. of hetero atoms (sulphate and βME)	13
No. of waters	584
R-factor (%)	14.6
R_{free} (%)	18.0
Average B_{iso} for protein atoms (Å^2)	15.8
Average B_{iso} for waters (Å^2)	27.4
Overall B_{iso} (Å^2)	17.2
RMS deviation for bond length (Å)	0.016
RMS deviation for angles (Å)	1.59

Figures within brackets are for the outer resolution shell of 1.69-1.62 Å.

$R_{merge} = \Sigma|I_i - I_m| / \Sigma I_i$ where I_i is the intensity of the measured reflection and I_m is the mean intensity of all symmetry-related reflections.

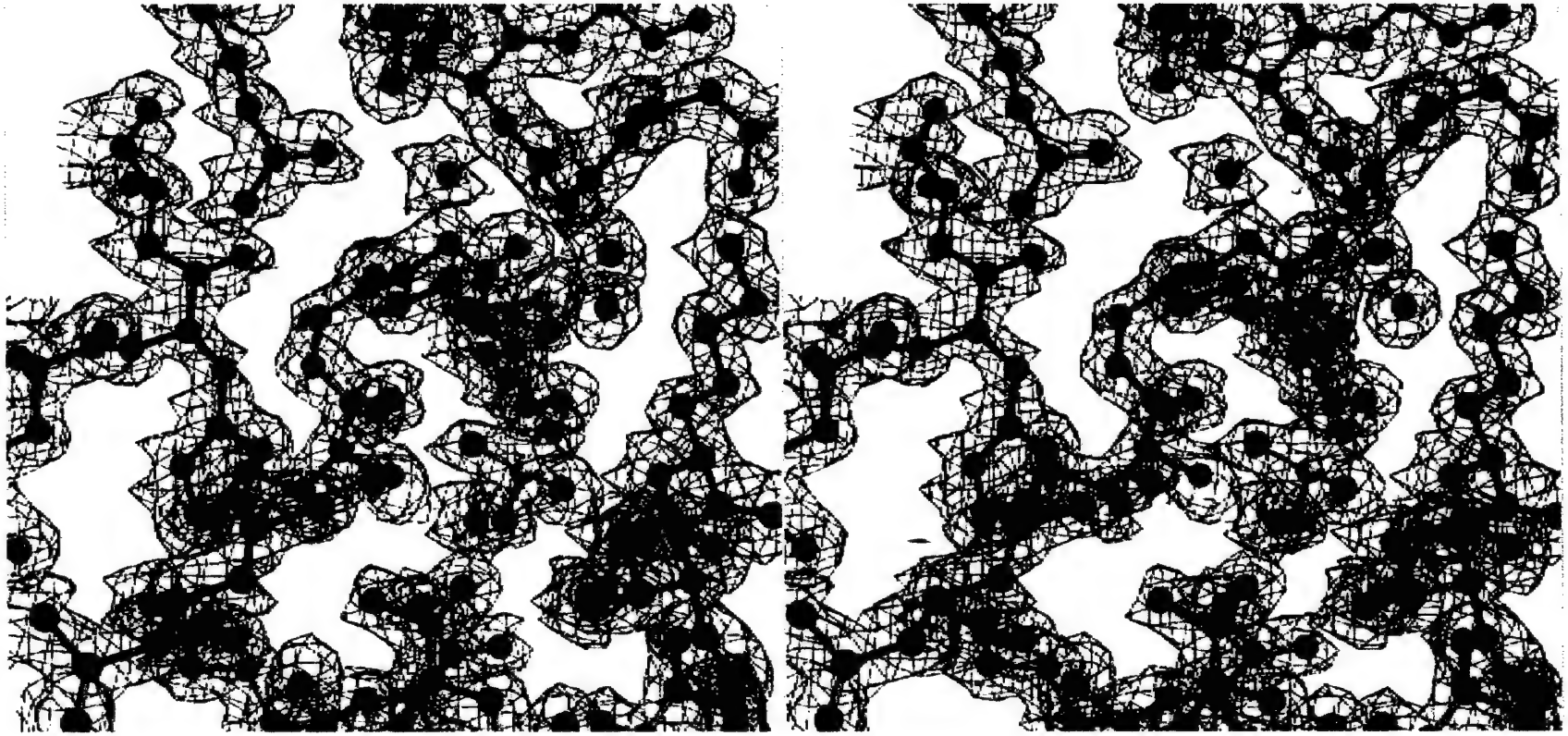

Figure 1. Stereoview of the electron density map of human phosphosphoglucose isomerase set against coordinates of the final refined structure, in ball and stick form. The area shown is the active site region. Bonds are coloured orange but those of the monomer generated by symmetry are coloured dark blue. Visible is this region is the bound sulphate ion (white bonds) and a molecule of β-mercaptoethanol (light blue bonds). The density is contoured at 1 σ. This Figure was produced using BOBSCRIPT.[62]

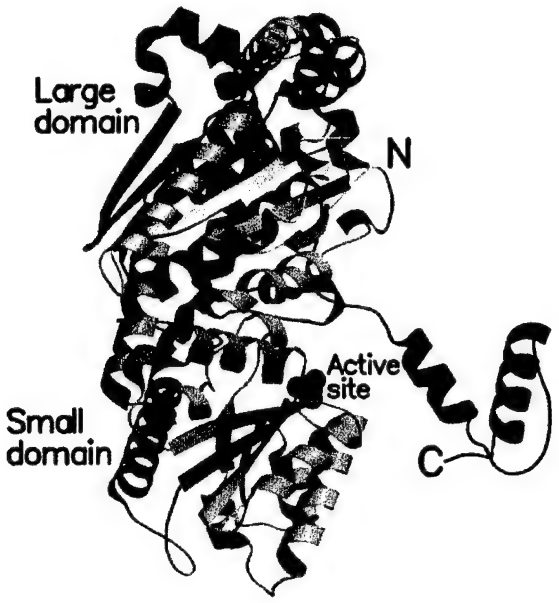

Figure 2. A ribbon representation of the structure of human phosphoglucose isomerase. The structure is colour ramped from blue at the N terminus to red at the C terminus. The active site is labelled and marked by the bound sulphate moiety, shown in CPK form. This Figure was produced using MOLSCRIPT.[63]

an αβα sandwich. The polypeptide chain begins in the large domain, crosses to the small domain and then returns to the large domain. The small domain contains a five-stranded parallel β-sheet surrounded on both sides by α helices. The large domain has a six-stranded mixed parallel/antiparallel β-sheet, also packed on both sides by α-helices. A striking aspect of the structure is a 45 residue extension at the C terminus which, in the dimer, wraps around the other monomer. On the opposite side of the molecule is another loop (residues 438-468), a "hook", that also interacts principally with the opposite monomer. It is likely that both of these features help contribute to the very high stability of the PGI in denaturing conditions.[24,25] The topology and assignment of secondary structure is shown in Figure 3.

Worthy of particular mention is the nature of the protein core between the β-sheet of the large domain and the packing helices α16, α17 and α22, two of which, α16 and α22, are almost completely buried within the structure. In addition to the normal hydrophobic interactions typically observed in protein cores, this region is characterised by a preponderance of markedly polar residues, water molecules, and, most notably, three charged residues, Asp355, Asp404 and Lys496, all of which are totally buried. The charges are balanced by forming compensatory electrostatic interactions with each other, and with His335, which also has the potential to be charged. Interestingly, one of the mutations associated with haemolytic anaemia is found in this region, G323S (see Table 2). This residue is on the core facing side of helix α23. A larger side-chain at position 323 would project into the tight core and disrupt many of the packing interactions.

The position of the active site in PGI is known from previous crystallographic studies of the pig enzyme bound to 5-phosphoarabinonate (5-PA),[26] the rabbit enzyme bound to 6-phosphogluconate (6-PG)[4] and, very recently, the structures of *Bacillus* PGI complexed with two inhibitors.[17] It is located in the cleft between the large and small domain and is close to the subunit boundary.

Comparison with other PGIs

The fold of PGI from *Bacillus* is essentially the same as PGI from mammals. The main differences are that the *Bacillus* enzyme has a shorter N-terminal region and that the hook structure is unexpectedly positioned on the opposite face on the molecule relative to the C-terminal "arm", whereas in mammalian PGIs these features are on the same side of the molecule. The active site regions are highly similar, concordant with these containing the most highly conserved residues.

Mammalian PGIs exhibit a high degree of sequence identity, typically 85-95%, and this is also reflected at the structural level. Crystal structures of three mammalian PGIs are now known (rabbit, pig and human) and all have essentially identical folds. For instance, the RMS deviation in all main-chain atoms between the human and pig enzymes is only 0.47 Å, with the only differences occurring in a few surface exposed loops. Greater differences are observed when comparing the human and rabbit structures. In this case the equivalent RMS deviations are 0.68 Å for monomer A and 0.60 Å for monomer B of the native rabbit enzyme (C.D. & H.M., unpublished data). Interestingly, the principal differences map to the active site region. In the human structure, the position of helix α13, together with its preceding connecting loop, lies much closer to the active site cavity than in either monomer of the native rabbit structure (Figure 4). Similarly, an adjacent loop between βc and α11 is also shifted away in the rabbit structure, although in this case the accompanying helix, α11, has shifted only slightly. The net effect of these differences is that, in the native rabbit enzyme, the active cavity is more open.

The human structure can also be compared with the structure of rabbit PGI containing the inhibitor, 6-PG.[4] The main-chain atoms of the human structure can be superimposed onto this structure with an RMS deviation of 0.517 Å for monomer A and 0.529 Å for monomer B, indicating again that overall, the two enzymes are highly similar. As before, the only significant difference is a slight shift in the relative position of α13. In both monomers of the 6-PG-bound enzyme, the position of this helix is intermediate between that observed in the human

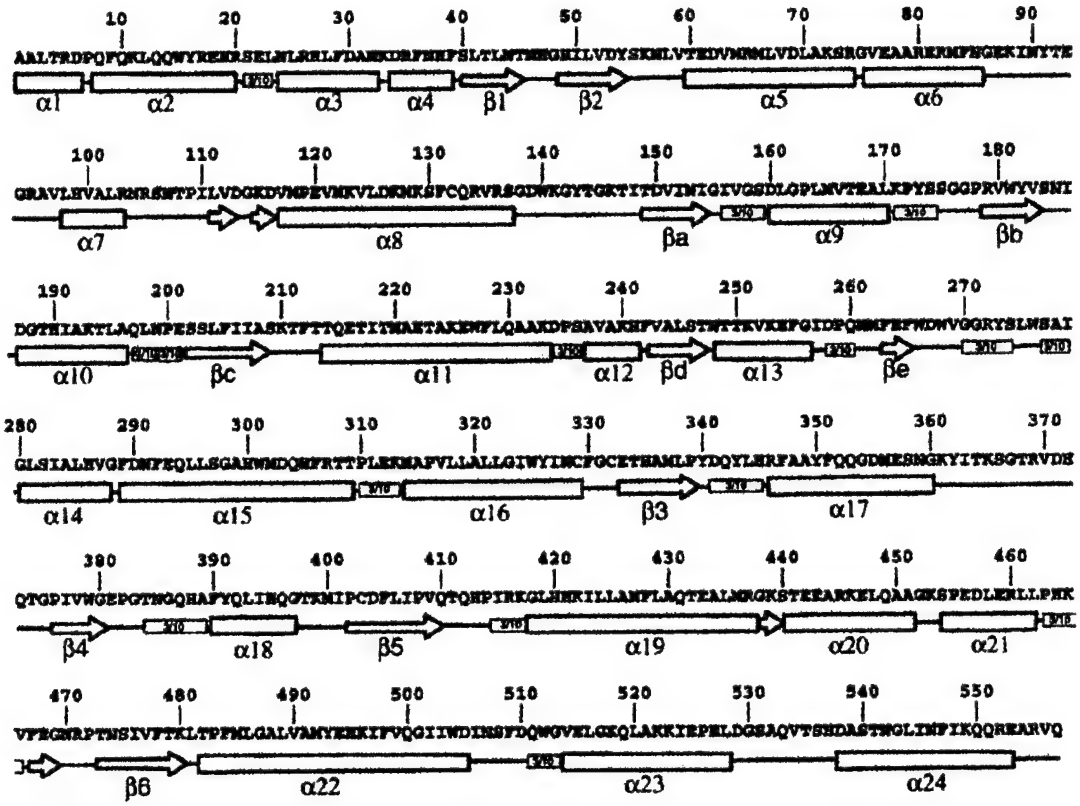

Figure 3. The secondary structure assignments of human phosphoglucose isomerase. The α helices are shown as boxes, β strands as arrows and sections of 3/10 helix are also shown as small boxes. The secondary structure was calculated using PROMOTIF.[64]

and unliganded rabbit enzymes, but is closer to the position in the human structure (Figure 4). The possible movement of this helix upon substrate binding is discussed below.

Active site

The high resolution of the human structure permits the most detailed view of the active site of PGI to date. Since the asymmetric unit in the human crystals is a monomer, the complete active site can only be visualized by generating a dimer using crystallographic symmetry. The active site comprises a number of residues that are totally conserved in all known sequences of PGI (Figure 5). These include Lys210, Gln353, Glu357, Gln511, Lys518 and His388# (# denotes from the other subunit, generated by symmetry). All of these point into the active site cavity and make few bonding interactions with other residues. The same residues have also been identified as active site residues in previous PGI structures.[3,4] At one end of the active site is a constellation of threonine and serine residues that, as the inhibitor-bound rabbit enzyme shows,[4] are responsible for binding the phosphate group. Other prominent residues include Arg272, which makes extensive contacts within the active site (see Discussion), glycine residues 157 and 158, and Ile156.

Although our structure is of the native enzyme in the absence of substrate, fortuitous binding of components from the crystallisation medium has helped provide a clearer indication of how the substrate may bind. One of these is a sulphate ion (from the precipitant ammonium sulphate) which has bound to the substrate phosphate-binding site. It is held in place by potential hydrogen bond interactions with threonine residues 211 and 214, serine residues 159 and 209, the main-chain nitrogen atoms of Lys210 and Thr211, as well as with several water molecules (Figure 6(a)). An additional threonine, Thr217, is also involved *via* hydrogen bonds to a water molecule.

Another component of the crystallization solution, β-mercaptoethanol, has also bound in the active site. This molecule extends across the active site with its hydroxyl group pointing toward the 157-158 loop and, at the other end, with its sulphide oriented towards the ε-amino group of Lys210. It makes only one direct contact with the enzyme, a potential hydrogen bond between its hydroxyl group and the main chain nitrogen of Gly158 (Figure 6(b)). Remaining contacts, to His388# and Lys210, are mediated through water molecules.

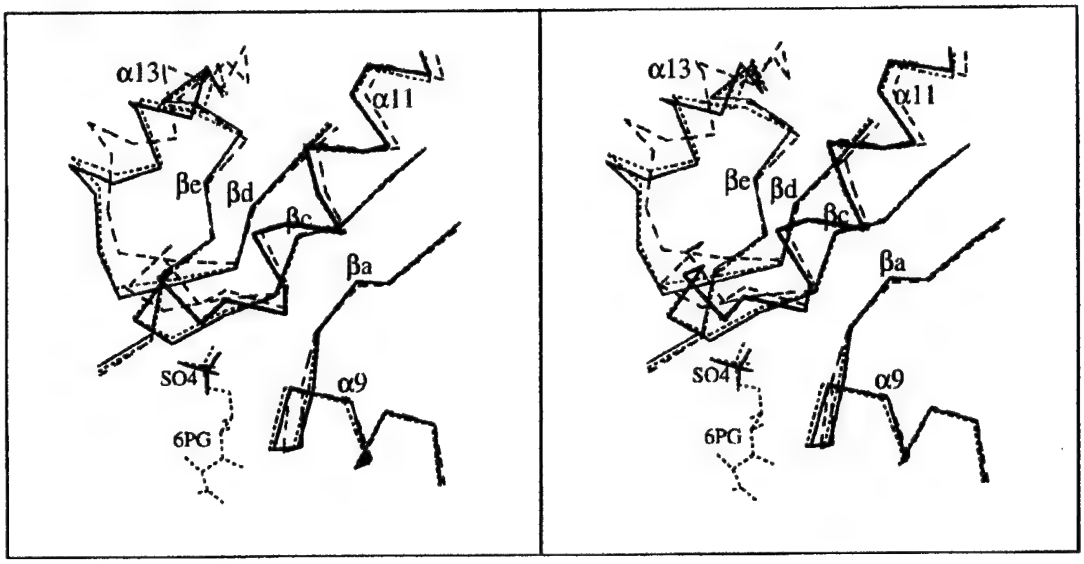

Figure 4. A comparison of human PGI with native and 6-phosphogluconate-bound rabbit PGI. Shown in this stereo representation is a close-up of a region in the small domain of PGI where most of the structural differences occur, specifically in the position of α13 and connecting loops. The human enzyme is represented by a solid line, the native rabbit enzyme (C.D. & H.M., unpublished data) by a long dashed line and the inhibitor-bound rabbit enzyme[4] as a short dashed line. Also shown is the sulphate ion, as observed in the human structure, and the 6-phosphogluconate in the inhibitor-bound rabbit structure. Note only one monomer of each rabbit structure was used for the superposition. This Figure was produced using MOLSCRIPT.[63]

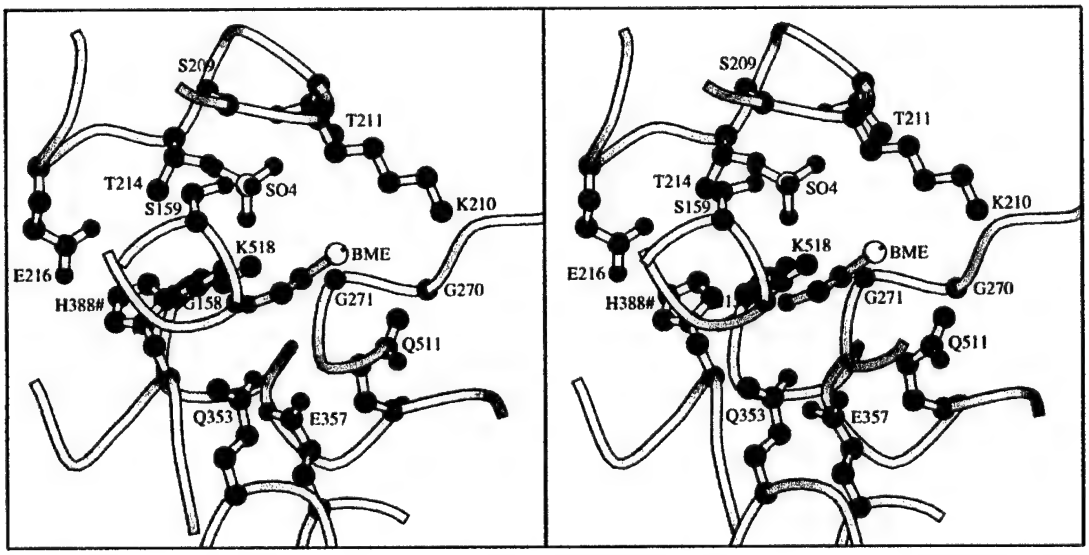

Figure 5. The active site region of human PGI. A stereo representation showing the residues forming the substrate-binding site, together with the bound sulphate and β-mercaptoethanol moieties, all shown as ball-and-stick. The other monomer in the dimer (coloured yellow) was generated by applying crystallographic symmetry to the monomeric asymmetric unit (coloured orange) (# denotes from the symmetry-related monomer). The bonds of the phosphate are coloured white and those of β-mercaptoethanol are coloured light blue. This Figure was produced using MOLSCRIPT.[63]

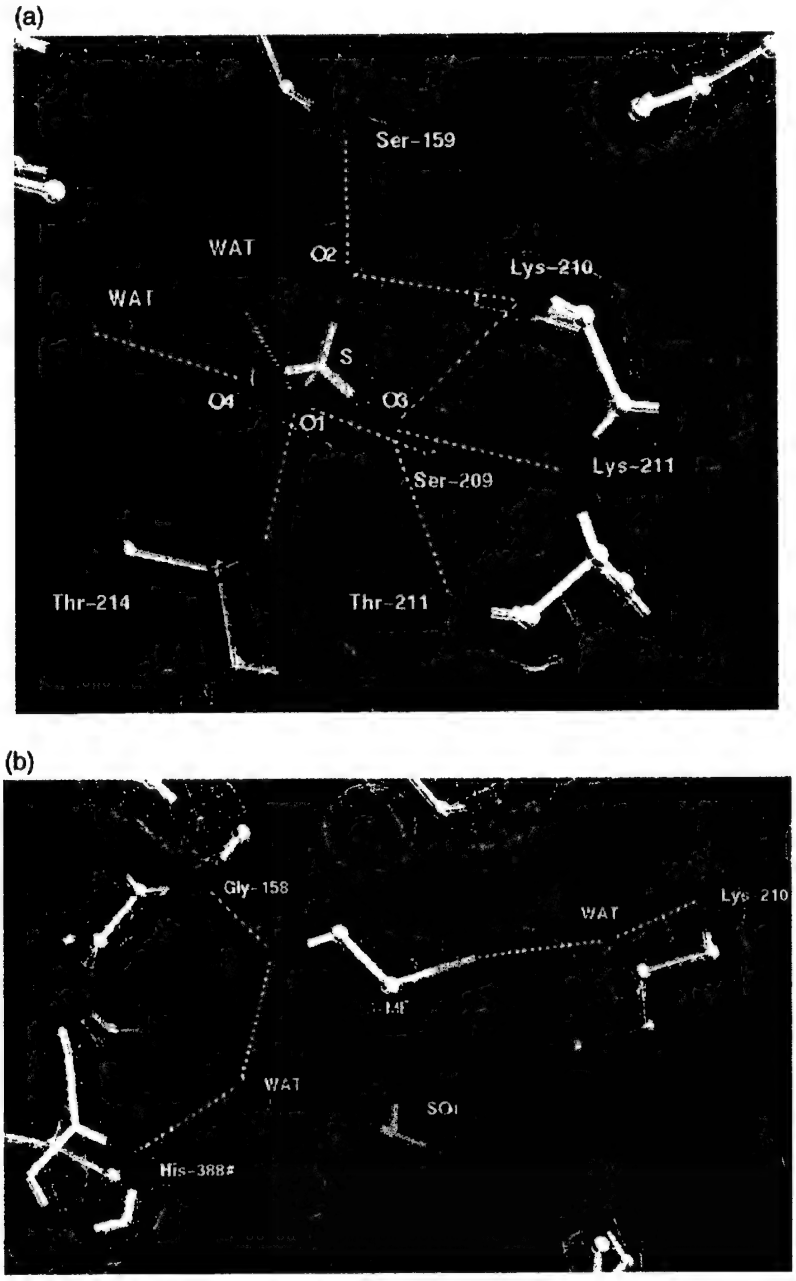

Figure 6. Electron density of the bound sulphate and β-mercaptoethanol molecules in the active site cavity. (a) A close-up of the sulphate binding site showing the potential hydrogen bonds (dashed lines) to the surrounding cluster of threonine/serine residues and water molecules. (b) The binding of β-mercaptoethanol, which makes few direct contacts with the enzyme. This Figure was produced using the O program.[54]

Discussion

Active site ligands

The active site of human PGI is populated by residues that are totally conserved in other species, including PGIs from bacteria. In Figure 7 a comparison of the active site of human PGI is made against inhibitor-bound rabbit and inhibitor-bound *Bacillus* enzyme. The active sites are indeed highly similar, especially between the mammalian enzymes. Although our structure is of the native enzyme, components of the crystallisation medium have bound to the active site, shedding light onto the possible mode of substrate binding. When compared with the inhibitor-bound rabbit enzyme,[4] there is a remarkable correspondence in the sulphate and phosphate positions (Figure 7(a)) show-

Figure 7. A comparison of the active site of human PGI with those of (a) 6-phosphogluconate-bound rabbit PGI (PDB code 1dqr) and (b) 5-phosphoarabinonate-bound *Bacillus* enzyme (PDB code 1c7r). For the human and *Bacillus* structures, the dimers were generated by applying crystallographic symmetry to the monomer in each asymmetric unit. In both of these stereo representations the backbone of the human enzyme is coloured yellow, the active site residues are red and the bound sulphate and β-mercaptoethanol moieties are shown in ball-and-stick form with the bonds coloured light blue. For (a) the backbone of the rabbit enzyme is coloured orange, the active site residues are green and the bound inhibitor is shown as ball-and-stick with magenta bonds. Alpha carbon positions of both enzymes are shown in cpk form and numbered (both in black). Note the high similarity between the two structures including the close overlap of the sulphate and phosphate positions. In (b) the backbone and active site residues of the *Bacillus* enzyme are coloured blue. The bound inhibitor 5-phosphoarabinonate is shown as ball and stick with magenta bonds. The alpha carbon positions of both enzymes are shown as cpk, yellow for human and blue for *Bacillus*, with red numbers for human and blue for *Bacillus*. Marked is the position of the sulphate molecule in the human enzyme and the phosphate group of the inhibitor in the *Bacillus* structure. Note how these are located in different positions, with the phosphate in the *Bacillus* enzyme being coordinated by an entirely different set of interactions at the lower part of the active site.

ing that the binding of sulphate has mimicked the phosphate group of the natural sugar substrate. As an example, the distance between O2 of the sulphate, or O2P of the phosphate, and the main-chain nitrogen atom of Lys210, is 2.84 Å in the former and 2.85 Å in the latter. Only one sulphate

oxygen atom (labelled O4 in Figure 6(a)) is non-bonded and this is likely equivalent to O6 in the sugar substrate. A nearby lysine residue, Lys210, and an arginine residue, Arg95, probably act to accommodate the negative charge of the phosphate group. The precise nature of the interactions of the sulphate/phosphate, involving serine and threonine residues and several water molecules, explains the high specificity of PGI for phosphorylated sugars.[27] Given this precision in both sulphate and phosphate binding, it is surprising to see that the phosphate group of 5-phosphoarabinonate (5-PA) has been positioned at the opposite end of the active site cavity in the structure of *Bacillus* PGI complexed with this inhibitor[17] (Figure 7(b)). As noted later, this has implications for the postulated reaction mechanism.

Converse to the situation with sulphate, the binding of β-mercaptoethanol in the active site cavity does not appear to imitate substrate binding. When compared to the position of 6-PG in the rabbit structure, β-mercaptoethanol lies in a perpendicular orientation and makes few specific contacts with the enzyme (Figure 7(a)).

Substrate-induced movement of active site loops

Our structure of human PGI can also be compared with two previously determined structures of PGI from rabbit: of the native enzyme (C.D. & H.M., unpublished data) and of the 6-PG inhibitor-bound enzyme.[4] Overall, the three structures are highly similar, indicating that few changes occur as a result of inhibitor binding. The only differences are seen in the positions of the helix α13 (and its preceding connecting loop) and the loop between βc and α11. In the inhibitor-bound rabbit structure both of these chains are significantly closer to the active site than in the native rabbit structure. Since the βc-α11 loop mediates most of the interactions for phosphate binding, its apparent movement toward the active site upon binding substrate is logical. The accompanying movement of α13 with the βc-α11 loop may be explained by the strong hydrophobic interactions between these two elements, in which Phe212 appears to have a central role. If these changes are indeed a direct consequence of inhibitor binding, they may reflect the structural rearrangement that has been postulated to be the rate-limiting step for catalysis.[28] Interestingly, in our human structure the positions of these loops are much closer to those seen in the 6-PG-bound structure. This is highly suggestive that one of the two molecules observed bound in the active site cavity of the human structure triggers the movement of α13 and the βc-α11 loop. Of these, it is more likely that the binding of sulphate is responsible, since it mimics the substrate phosphate. These structural data imply that recognition of the phosphate group alone is sufficient to promote the "active" conformation of the enzyme after substrate binding. Structural movements arising from differences in crystal packing are unlikely, since both rabbit structures are solved from the same crystal form.

Complicating the picture is evidence that the various active site inhibitors of PGI may bind differently. In the recently published structure of *Bacillus* PGI complexed with 5-phosphoarabinonate,[17] not only does the inhibitor lie in a different orientation to that of 6-PG in the rabbit structure, but a different part of the active site has moved as a result of binding inhibitor (Figure 7(b)). In this case residues 200-204, equivalent to 270-274 in mammals, have moved closer, apparently to bind with the phosphate group. In the human structure, this region is the active site loop between βe and α14, containing two stretches of 3/10 helix. When compared to the two rabbit structures, however, this part of the structure shows no evidence for movement upon binding inhibitor. In the accompanying structure of *Bacillus* PGI bound to *N*-bromoacetylethanolamine phosphate (BAP),[17] the picture is closer to that seen in mammalian PGIs: the orientation of the bound inhibitor is the same as 6-PG in the rabbit enzyme and equivalent conformational changes occur (not shown).

It is clear that structural studies of PGI bound with the various inhibitors need to be carried out at a much higher resolution to enable a fuller understanding of the mode of substrate binding and of any consequent conformational changes.

Reaction mechanism

The isomerisation reaction is postulated to proceed by general acid/base catalysis *via* a *cis*-enediolate intermediate, generated by proton abstraction from C1 (for F6P) or C2 (for G6P) (see ref. 1). Evidence for such an intermediate comes from early experiments showing exchange of a proton with solvent[29] and the observation that inhibitors of PGI mimic a *cis*-enediol structure.[30] Since the acyclic forms of glucose 6-phosphate and fructose 6-phosphate are present in solution in only trace amounts,[31] the enzyme is also presumed to catalyse ring opening. In support of this, PGI has an inherent anomerase activity that is catalytically distinct from the isomerisation reaction.[32] Chemical modification studies have suggested several candidates for residues having a role in catalysis, including lysine,[33,34] arginine,[33,35] histidine,[36] glutamate[37] and tryptophan,[33] and all of these residues are found in the active site pocket. Observed pK_a values of 6.75 and pH 9.3 led researchers to propose that lysine and histidine residues were central to the reaction mechanism;.[2] the lysine to catalyse ring-opening and the neutral imidazole group as the base for proton abstraction. Based on the crystal structure of rabbit PGI bound to 6-PG, one group has proposed that Lys518 and His388 act as a general acid and base catalyst, respectively.[4] Central to this hypothesis is the role of Glu216, which forms a charge couple with His388 and may act to increase its basicity. How-

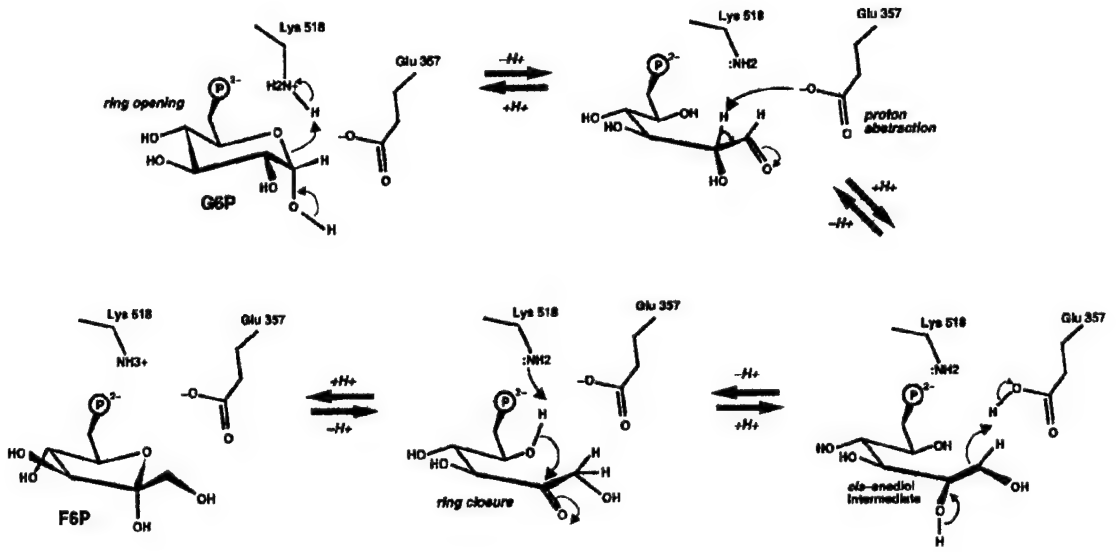

Figure 8. A proposed reaction mechanism for phosphoglucoseisomerase. In this scheme Glu357 is the base responsible for proton abstraction from the C1 and C2 positions of fructose 6-phosphate and glucose 6-phosphate respectively. After substrate binding, the first step is ring opening, which here is shown to be catalysed by the acid group Lys518, but could equally be likely catalysed by His388. This results in the loss of a proton from the C1 hydroxyl group to the solvent, forming a carbonyl group. Glu357 then abstracts a proton from the C2 position of G6P, causing electrons to flow towards the C1 carbonyl. The resulting negative charge attracts a proton from the solvent, forming the *cis*-enediol intermediate. Glu357 then donates back a proton to the C1 position. The resulting electron flow towards Glu357 leaves a carbonyl group at C2. In the final step Lys518 (or His388) abstracts a proton from the sugar ring oxygen leading to ring closure and the reestablishment of a hydroxyl group at C2.

ever, in this structure both His388 and Lys518 are too far away to interact directly with the C1 and C2 positions of the 6-PG inhibitor, requiring that the true substrate must bind somewhat differently. The structure of *Bacillus* PGI bound with 5-PA has been interpreted to propose an alternative scheme in which Lys420 (equivalent to Lys518 in mammals) acts as a base for ring opening, His308 (His388) is the base responsible for proton abstraction, and Glu285 (Glu357) acts as a general acid by donating a proton to the C1 carbonyl group.[17] The active site glutamate residue was identified by using the inhibitor 1,2-anhydro-D-mannitol-6-phosphate.[37] With the orientation of 5-PA in this model, His308 is indeed well placed to act as the base at C1/C2. However, the existence of two binding modes for PGI inhibitors, that this model necessitates, is hard to reconcile both with the absolute specificity of PGI for phosphorylated sugars[27] and the highly precise interactions of the sulphate moiety observed in our human structure.

In the rabbit 6-PG structure Glu357 is better placed than His388 to abstract a proton from the C1 and C2 positions of the inhibitor. Moreover, the choice of a bidentate residue for a suprafacial 1,2 hydrogen transfer is a logical one. We introduce, therefore, a third possible scheme in which Glu357 is the base catalyst (Figure 8). As such it may be similar to the role Glu165 plays in triosephosphate isomerase (TPI)[38] where the position of the carboxylate group with respect to C1 and C2 carbon atoms of the substrate is critical. As with Glu165 in TPI, the pK_a of Glu357 must be raised by two to three pH units for it to act as an effective base. How this is achieved is uncertain, particularly with the adjacent arginine residue (Arg272), though it may be a result of this residue being buried in a hydrophobic environment. Gln511 is positioned such that it may have a role in the mechanism by stabilising the *cis*-enediol intermediate *via* hydrogen bonding interactions. The nature of the residue responsible for ring opening is more questionable. One possibility is Lys518, *via* a general acid mechanism in which its proton is donated to the ring oxygen atom.[2] Alternatively, His388 may serve as a base catalyst for ring opening: its basicity being enhanced by the charge couple with Glu216. There is good evidence that His388 acts as a base in catalysis,[39] but since these experiments have not separated the isomerase and anomerase activities of PGI, it could equally likely act as a base in ring-opening. Whatever its precise role, His388 is clearly an important residue, as evidenced by its mutation to Ala, Asn and Gln leading to a 1000-fold lower isomerase activity of *Bacillus* PGI[39] and the existence of the Calden variant of haemolytic anaemia.[40] The role of Arg272 may be to stabilize the negative charge on the enediolate intermediate, as has been proposed.[4] Alternatively, by making numerous contacts with other components of the active site (see Figure 9(b)), it may serve a structural role by maintaining the correct architecture of the active site, including the position of Glu357.

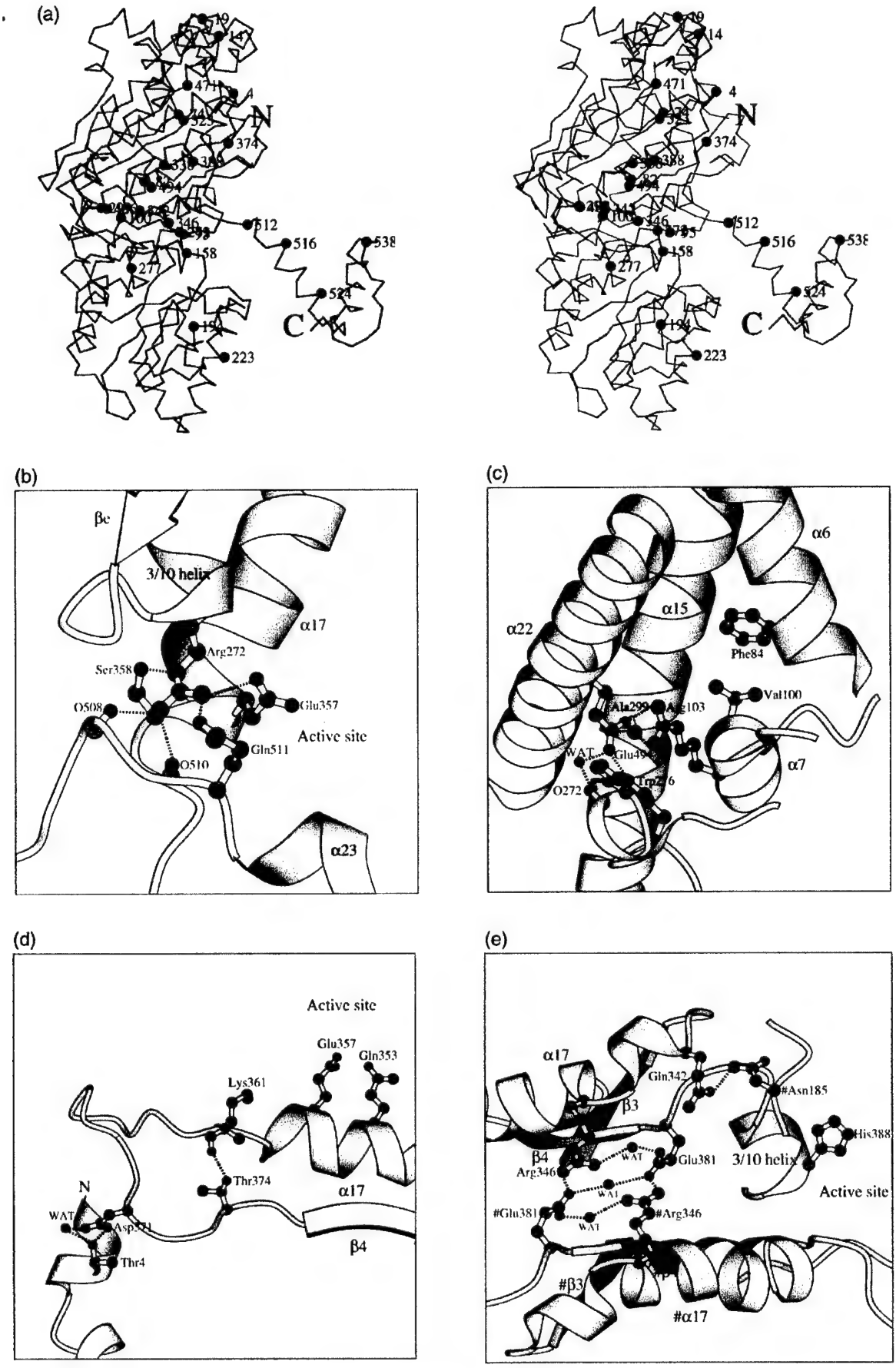

Figure 9 *(legend shown on page 458)*

Which of these mechanisms is shown to be correct, or indeed whether a wholly different mechanism is at play, awaits more detailed studies. The presence of multiple charges in the active site of PGI increases the potential for charges to be delocalised over more than one ionizable group, resulting in cooperative pK_a effects, and complicating any proposed mechanism.

Mutations associated with haemolytic anaemia

PGI is an essential enzyme and its inactivation in mice is embryonic lethal.[41,42] PGI deficiency in humans is an autosomal recessive genetic disorder resulting in nonspherocytic haemolytic anaemia. Many of the mutations in PGI associated with haemolytic anaemia have now been characterised at the molecular level (for a review, see ref. 19). These mutations are all homozygotes or compound heterozygotes of partially inactive enzyme alleles. Although the potential effect of some of these was discussed previously in the context of the rabbit enzyme,[4] the high-resolution structure of the human enzyme is more relevant for their understanding, especially where contacts are mediated *via* water molecules. The roles of these residues and the likely effect that their mutation has had upon the PGI structure has been examined in detail (Table 2). The distribution of the mutations within the PGI fold is shown in Figure 9(a). The mutations can be classified loosely into three groups: (a) those that impact the precise structure of the enzyme, S; (b) those that disrupt or alter a dimer-dimer contact, D.I.; and (c) those of residues at the active site, which may have a role in catalytic function, A.S. Many of these mutations illustrate just how critical the precise three-dimensional structure is for correct function. The majority of the mutations disrupt key interactions that contribute directly or indirectly to the active site architecture. Some of these are shown in Figure 9. The importance of the two 3/10 helical segments in the active site (residues 270-274 and 277-279) is illustrated by several different variants. The Arg272His mutation (Figure 9(b)) would remove an essential residue that makes contacts between the first 3/10 helix and two other active site components, the loop between helices α22 and α23, and helix α17. These elements contain residues that point into the active site and likely have a role in substrate binding or catalytic function (see above). In particular helix α17 is highly conserved: one side of this helix contains Glu357 and Gln353, both of which project into the active site. The other side of this helix packs into the atypical protein core, described earlier, in which Asp355 has a prominent role. The mutations Val100Met, Glu494Lys and Ala299Pro also potentially disrupt the active site architecture by altering the critical interactions between helices α22, α7, and α6 and including the 3/10 helix between residues 270 and 274 (Figure 9(c)). The position of the active site helix α17 is likely to be altered as a result of two other mutations, Thr374Arg and Thr4Ile (Figure 9(d)). The mutation at 374 would break the hydrogen bond from the threonine side-chain to the carbonyl group of Lys361, which links β4 and α17. The Thr4Ile mutation would remove the potential hydrogen bond between the N-terminal helix and the α17-β4 loop. An example of a critical dimer-dimer interaction is provided by the Arg346Cys mutation, which occurs in several variants (Figure 9(e)). This residue forms an electrostatic interaction across the dimer interface with Glu381# and, *vice versa*, Arg346# interacts with Glu381. There is also a network of water molecules linking all four residues. Nearby is a second mutation, Gln342Arg, which would also disrupt a dimer interface interaction, with Asn185#. As seen in Figure 9(e), the active site is very close to the sites of both of these mutations.

Two of the mutations are of active site residues and fall into the last category. The Gly158Ser mutation would lead to a bulkier side-chain projecting into the active site and would probably restrict substrate binding (see Figure 5). Alternatively, removal of the glycine residue may reduce the conformational flexibility of the active site loop that is highly populated with glycines (residues 155-158). His388 almost certainly has an important role in catalysis (see above) and its replacement by an arginine would lead to reduced catalytic function (see Figure 5).

How does PGI act as a cytokine?

In order to decipher how PGI acts as a cytokine, there are three key questions to be answered: (1) what exactly is the nature of the cytokine version of PGI; (2) how does the PGI cytokine bind to its putative receptor and (3) how is PGI secreted without a leader peptide? Answering these reveals contradictions and ambiguities that remain to be resolved.

On the first of these questions, the cytokine version of PGI has been reported to be a monomer of

Figure 9. Residues in phosphoglucoseisomerase whose mutation is associated with haemolytic anaemia. (a) The fold of a single monomer of PGI shown as a backbone trace with the C^{α} positions of the mutations (listed in Table 2) plotted as circles. (b)-(e) The structural roles of selected residues. In each case the mutated residues are coloured yellow and the potential interactions they make are denoted by dashed lines. The approximate location of the active site is noted. The chain of the asymmetric unit is coloured blue and, where shown, that for the symmetry-generated chain is coloured orange. (b) Arg 272; (c) Glu494, Val100 and Ala299; (d) Thr4 and Thr374; and (e) Arg346, Gln342 and His388. This Figure was produced using MOLSCRIPT.[63]

Table 2. Mutations in the human PGI associated with haemolytic anaemia and their likely effect on the structure

Variant	Ref.	Mutation	Type	Role in structure	Likely effect of mutation
Matsumoto	a	Thr4Ile	S/A.S.	H bond with D371 (Figure 7(c))	Alter packing of N term and α17-β4 loop
Stuttgart	b	Gln14Stop	S	n/a	Truncation
		Gln342Arg	D.I./A.S	H bond with #N185, HB contact with #H190	Break link across dimer interface near A.S.
Homburg	c	His19Pro	S	E.S. contact or H bond with E22	Alter α2-α3 connection
		Leu338Pro	S	Part of β3	Disrupt β sheet in large domain
Elyria	d	Arg74Gly	S	H bonds with D505, D7 (via H20) & O of 502	Break link between α1, α5 & α22
		Arg95Stop	S	n/a	Truncation
*	e	Arg82Trp	S	E.S. with E87 & H bond with O of 88	Alter α6-α7 surface loop
Bari	f	Arg95Stop	S	n/a	Truncation
		Thr194Ile	S/D.I.?	H bonds to O's of 190 & 183 (via H2O)	Break β3-α10 contact (α10 is D.I. helix)
*	d	Arg95Stop	S	n/a	Truncation
		Arg346Cys	D.I.	Salt-bridge with #E381 (Figure 7(d))	Break α17-#β4 link across dimer interface
Sarsina	f	Val100Met	S	Hydrophobically packs against F84 (Figure 7b)	Alter packing between α6, α7 & α15
*	g	Gly158Ser	A.S.	On active site loop near residues 271-172	Larger s/c would change A.S. architecture
		Arg346His	D.I.	see Arg346Cys	Break α17-#β4 link across dimer interface
Mola	f	Thr194Ile splice site	D.I.	see Bari	Break β3-α10 contact (α10 is D.I. helix)
Iwate	a	Thr223Met	D.I.	H bond to #R417	Breaks α11-#α19 link across D.I.
*	e	Thr223Met	D.I.	see Iwate	Breaks α11-#α19 link across D.I.
		Glu494Lys	S/A.S	S.B. with R103 & H bond to W276 (Figure 7(b))	Alter packing between α7, α22, & A.S 3/10
*	e	Arg272His	A.S.	Numerous hydrogen bonds in A.S> (Figure 7(a))	Alter active site architecture
		Arg346Cys	D.I.	see above	Break α17-#β4 link across dimer interface
*	e	Ser277Leu	S/A.S.	H bonds to N of 279 & O of 274	Alter architecture of key A.S. loop
		Leu486Phe	S	HB core residue between L & S domains	Bulkier residue would alter α22/α9 packing
*	d	Ala299Pro	S	Packs against R103 and W276 (Figure 7(b))	Kinks α15 helix that buttresses A.S. loop
		Arg346Cys	D.I.	see above	Break α17-#β4 link across dimer interface
*	b	Gly323Ser	S	Residue of buried helix α16	Disrupt packing against L domain β sheet
Morcone	f	Gln342Arg	D.I./A.S	see Stuttgart	Break link across dimer interface near A.S.
Narita	a	Gln342Arg	D.I./A.S	see Stuttgart	Break link across dimer interface near A.S.
Nordhorn	h	Gln342Arg splice site	D.I./A.S	see Stuttgart	Break link across dimer interface near A.S.
Mt. Scopus	d	Arg346Cys	D.I	see above	Break α17-#β4 link across dimer interface
Zwickau	h	Arg346Cys	D.I.	see above	Break α17-#β4 link across dimer interface
		Trp512Stop	D.I.	C term region makes many dimer contacts	Lose many dimer-dimer contacts
Kinki	a	Thr374Arg	S/A.S.	H bond to O and N atoms of K361 (Figure 7(c))	Alter conformation of active site α16
		Asp538Asn	S/D.I.	N cap of α24 & HB with #M436 & #F29	Destabilise α24 and weaken D.I. contact
Calden	c	His388Arg	A.S.	A.S. residue with possible cataytic role	Alter catalytic properties
		Leu516Val	D.I.	Packs between #A434 & #F467	Changes D.I. H.B. packing near A.S.
*	d, b	Arg471His	S/A.S.	H bond to O's of 395, 394 & 469 & E470(wat)	Disrupt link between LEL and α18
*	g	Ile524Thr	D.I.	Packs against #L429 and #A430	Disrupts D.I. α23 & #α19 HB packing
Fukuoka	a	Asp538Asn	S/D.I.	see Kinki	Destabilise α24 and weaken D.I. contact

*, variant has no name; S, structural; D.I., dimer interface; A.S., active site; HB, hydrophobic; #, symmetry-related; ES, electrostatic; LEL, large external loop; References: a;[65] b, W. Kugler, P. Laspe & M. Lakomek, 1999, unpublished; c;[40] d;[66] e;[67] f;[68] g;[69] h.[70]

approximate molecular mass 55 kDa.[5,7,8,43] This is at odds with biochemical and structural data showing that PGI exists as a dimer with a subunit mass of 63 kDa. As the available crystal structures show, PGI is a tight dimer with numerous and highly specific contacts between the subunits. Two structural features, the hook between residues 438 and 469 and C-terminal extension, both of which wrap around the partner monomer, likely contribute to the high stability of the enzyme. Moreover, it is well established that the dimer is necessary for catalytic function[16] and the structure shows very clearly why this is the case. In a monomeric enzyme the contribution of His388 from the other subunit would be absent and it is likely that the resulting protein would be, at the very least, catalytically impaired. The importance of the dimer, at least for catalytic function, is also suggested by those instances of haemolytic anaemia arising through mutations of residues mediating dimer-dimer contacts. Also at odds with the existence of a monomeric cytokine PGI is the evidence that AMF,[6,7] neuroleukin,[44] MF[7] and MBSPI,[8] all manifest PGI activity. Indeed several of these reports also demonstrate that the commercially available rabbit muscle enzyme itself possesses cytokine activity. Furthermore, two recent papers reveal that enzymatically active recombinant PGI has full autocrine motility factor activity.[45,46] Given this evidence, it is hard to envisage PGI existing as anything other than a dimer, suggesting that smaller or monomeric forms of the protein may not be directly related to cytokine activities. Nevertheless it will be essential to establish unambiguously the true molecular mass and oligomeric state of the cytokine versions of PGI.

To function as an extracellular cytokine PGI must bind to a receptor. One group isolated a cDNA clone for a putative AMF receptor using a monoclonal antibody with AMF-agonistic activity.[15] The receptor was more recently described as a putative seven transmembrane domain protein.[47] The high-resolution structure of PGI, however, gives few clues as to how this enzyme may bind to such a receptor. There are no striking hydrophobic areas on the surface of the enzyme, and since the protein is so well conserved overall within mammals, clusters of highly conserved residues on the surface cannot readily be identified. An alternate possibility is that if the cytokine PGI is indeed a monomer, the remnant active site mediates receptor binding, perhaps by binding to sugar moieties on the glycoprotein receptor molecules that are similar to the substrate sugars.[48] It should be noted, however, that PGI binds sugar substrates with millimolar affinities, whereas the cytokine activities of PGI are observed at protein concentrations of less than nanomolar. The main contribution from the adjacent monomer to the active site is the 3/10 helix (residues 384-389). Were this to be removed, the active site would be more exposed but most of the residues responsible for binding the substrate would remain. Indeed, matrix-bound monomers of PGI can still bind substrate.[16] In support of this hypothesis, several specific inhibitors of PGI activity, which bind to the active site, also abolish AMF-induced cell motility with no effect on basal migration,[6,17] suggesting that there is structural overlap of the regions responsible for the catalytic and cytokine functions of PGI. The alternative explanation is that these inhibitors may induce subtle conformational changes that alter binding to the receptor. The fact that PGI from *Bacillus* manifests AMF activity also argues for a role of the active region in cytokine function,[3,17] since the most conserved regions lie at the active site. Identification of the precise epitope responsible for receptor binding is a major priority for future investigations. No detailed biochemical studies of ligand-receptor binding have yet been published, but a first step is the recent demonstration of specific binding of biotinylated rabbit PGI to intact mammalian cells.[49]

The third question concerns the mechanism by which PGI is secreted from the cell. PGI lacks a leader sequence and is probably released directly from the cytoplasm. Cell lysis and necrosis could lead to release, but a non-classical secretory pathway may also be involved.[50] One report has linked the secretion of AMF with the phosphorylation of Ser183.[46] This residue is located at the base of a deep cleft, so it is hard to envisage a protein kinase gaining access to such a buried residue. Whatever the mechanism, given the resistance of folded PGI to dissociation and denaturation, it is likely that the active dimer is secreted intact across the plasma membrane.

Materials and Methods

Protein expression and purification

A 1.7kb DNA fragment encoding human PGI was prepared by PCR in a manner exactly parallel to that described for the rabbit enzyme.[51] Flanking PCR primers changed the 5′ end, so that the methionine start codon was within a unique *Nde*I restriction enzyme recognition site, and so that the stop codon was replaced by six histidine codons followed by a new UAA stop codon and an *Eco*RI recognition site. The PCR primers were used to amplify the open reading frame of human PGI using minimal cycle number and high fidelity Vent DNA polymerase (NE BioLabs Inc.). Template was the human adult brain cDNA clone 184111 (IMAGE Consortium) identified from a BLAST search of the human EST database as EST H30758. The amplified DNA was subcloned into the bacterial expression vector pET5a as an *Nde*I to *Eco*RI fragment and expressed in *Escherichia coli* BL21DE3pLysS (Stratagene Inc.). The bacterial cultures were induced with 0.5 mM i-PTG for three hours at 30 °C. Cell pellets were collected by low speed centrifugation, lysed by sonication without protease inhibitors, and clarified by high speed centrifugation. The soluble supernatants were bound to NiNTA agarose (Qiagen Inc) and washed and eluted according to the manufacturer's standard protocol. The material eluting in 0.25 M imidazole was >95% homogenous as estimated from

Coomassie blue staining of the protein on denaturing, reducing 12.5% polyacrylamide gels. Recovery was approximately 50 mg per litre of bacterial culture. PGI was concentrated and equilibrated with phosphate-buffered standard saline using Centricon-30 ultrafiltration (Amicon). The purified protein was stable, had kinetic properties very similar to those of the rabbit muscle enzyme, and was free of detectable bacterial endotoxin (Hill, Li, and Chirgwin, in preparation).

Crystallisation

Using Centricon microconcentrators (Amicon), the protein was exchanged into buffer containing 15 mM Hepes at pH 7.5 and 2 mM β-mercaptoethanol and concentrated to approximately 4 mg/ml. Crystals were grown by the hanging drop method in which 3 μl of well solution was mixed with 3 μl of the protein solution. A variety of crystallisation conditions were tested.

Data collection

Crystals of hPGI were cryo-frozen by passing through a solution containing 100 mM Tris (pH 8.5), 2.6 M ammonium sulphate and 30% (v/v) glycerol, added as cryoprotectant. Diffraction data extending to 1.62 Å resolution were collected at Daresbury SRS, station PX7.2 (wavelength = 1.488 Å) on a Mar 345 image plate. The data were collected in two passes. For the low-resolution data the crystal-to-plate distance was 222.7 mm and 50 images were collected each with an exposure time of two minutes per 1° oscillation frame. For the high-resolution pass the crystal-to-plate distance was 120 mm and 100 images were collected with an exposure time of two minutes per 0.6° oscillation frame. The data were integrated using DENZO and scaled with SCALEPACK.[52]

Structure determination

The structure of hPGI was solved using the refined structure of pig muscle PGI (C.D. & H.M., unpublished results). After replacing the sequence to correspond to that of human PGI, the initial model was subject to several cycles of rigid body refinement followed by a round of conventional refinement, both using XPLOR.[53] Further improvements to the model were made by cycles of manual rebuilding, using O,[54] and crystallographic refinement with REFMAC.[55] Water molecules were added using ARPP[56] and checked manually. Since the first residue of native PGI from rabbit is known to start with an alanine residue,[57] the final model is numbered 1 to 557. Incomplete density was observed for the non-native methionine residue at the N terminus and so was omitted from the model.

In published sequences of human PGI there is a conflict at position 157, with one entry listing a glycine[58] whereas others show a valine[59,60] (accession K03515, M. Gurney, unpublished). Notably, in other species of PGI this residue is totally conserved as a glycine. When refined as a valine, $|F_o| - |F_c|$ difference maps revealed a large peak of negative density around the side-chain and the phi/psi angles also fell within a disallowed region of the Ramachandran plot. We have therefore modelled this residue as a glycine residue.

Structure superimpositions were performed using the CCP4 program LSQKAB.[61] In the case of the rabbit enzyme (PDB code 1dqr) all atoms were included, whereas for the *Bacillus* structure (PDB code 1c7r) only active site loops (with side-chains) were included.

Protein Data Bank accession codes

The coordinates have been submitted to the RCSB with accession code 1IAT.

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Appendix B

Title/Referral Page

No Page Limit

1. Proposal title (up to 160 characters)

TUMOR-SECRETED AUTOCRINE MOTILITY FACTOR [AMF]: CAUSAL ROLE IN AN ANIMAL MODEL OF CACHEXIA

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TUMOR-SECRETED AMF: CAUSAL ROLE IN AN ANIMAL MODEL OF CACHEXIA

Technical Abstract: J.M. Chirgwin, Ph.D.

Background: **Autocrine motility factor** (AMF) is expressed by human breast cancer cells, such as MCF7, where its mRNA is stimulated by heregulin. Also known as phosphoglucose isomerase [PGI] and neuroleukin, it has been used as a serum marker of metastatic breast cancer for 50 years. We recently found that tumor cells constitutively secreting AMF caused periosteal new bone formation in two different models of bone metastasis. Animals with significantly increased serum AMF concentrations displayed tumor-associated weight loss (cachexia), a major cause of morbidity and mortality in advanced disease. The cell line engineered to secrete mouse AMF, when grown as an intramuscular tumor, caused severe cachexia in mice, while control tumors were larger but caused no cachexia. This provides strong evidence that **AMF is a novel tumor cachectic factor**: a role consistent with the extensive clinical literature describing AMF/PGI as a serum marker of advanced metastatic disease.

AMF binds to cell surface receptors. We found that AMF induced expression in bone marrow stromal cells of RANK ligand, a potent bone-resorbing factor. The responses displayed a 100-fold species-specificity for mouse versus human AMFs. Despite its clear importance in breast cancer, the mechanisms of action of AMF via receptor binding are under-investigated. AMF is the secreted form of an intracellular glycolytic enzyme, phosphoglucose isomerase, but the relationship between extracellular and intracellular forms of the protein is controversial.

Objectives & Specific Aims: We propose a series of specific aims to answer five outstanding questions about the induction of cachexia by AMF and the binding of AMF ligand to its receptor:

1) **Is recombinant AMF protein sufficient to cause cachexia in vivo?** Recombinant mouse AMF will be delivered continuously to mice via mini-pumps. The first experiment will determine the dosage to achieve blood levels equivalent to those seen in mice with tumor-induced cachexia. AMF may act through inflammatory cytokines. We will test for increases in the cachexia-associated cytokines, IL-1, IL-6, IFN-γ, and TNFα, in AMF-treated mice.

2) **Is isomerase catalytic activity required for AMF-induced cachexia?** Inside the cell PGI interconverts glucose and fructose 6-phosphates in the glycolytic pathway. It is unclear whether this enzymatic activity is necessary for the extracellular cytokine functions of the protein. We will make 2 mutants of AMF/PGI: E357A, which lacks the side-chain responsible for catalytic proton transfer, and S209T211,214,217/4A, which eliminates the groups needed to bind the phosphate group of the substrate. Experiments will be guided by our 1.6Å x-ray structure of the human enzyme. Mutant proteins will be assayed in vitro for isomerase activity and receptor binding and in vivo for ability to cause cachexia.

3) **Are the cachectic actions of AMF species-specific?** Bone cells in vitro show a 100-fold preference for AMF of the same species when mouse and human are compared- predicting that much higher concentrations of human AMF than mouse AMF will be required to cause cachexia in mice. We will test this prediction as in Aim 1.

4) **Does AMF act via a high-affinity cell-surface receptor?** AMF shows a bell-shaped dose response curve in vitro and in vivo, suggesting an activation mechanism in which receptor is dimerized by low, pM concentrations of dimeric ligand. We will test this mechanism by biochemical binding studies in vitro using [^{125}I] mouse and human AMF ligands and mouse and human cell lines.

5) **Can cachexia be alleviated by treatment with monoclonal antibodies which prevent AMF binding to its receptor?** We have raised a panel of monoclonal antibodies [mAbs] against recombinant AMF/PGI. We will test the individual antibodies for their ability to inhibit ligand binding as in Aim 4. Blocking antibodies will be tested in vivo, as time permits, for their ability to decrease cachexia induced by recombinant AMF/PGI as in Aim 1.

Study Design: Various forms of AMF will be expressed in *Escherichia coli* and purified to homogeneity. They will be tested for binding to cells and by enzymatic assay in vitro. AMFs will be continuously infused into mice via implanted Alzet mini-pumps. AMF concentrations will be assayed in the circulation and cachectic actions determined from body weight and histology of kidney, spleen and liver after sacrifice.

Relevance: All of the technology for the proposed work is proven and available in the P.I.'s laboratory. Recombinant proteins, including a high resolution crystal structure of human AMF, have been generated, as well as a reproducible animal model for tumor-induced cachexia. Actions of AMF have previously not been studied in vivo. The binding of AMF to its receptor offers a target for development of therapeutics, such as the mAbs to be tested in Aim 5, to decrease the morbidity and mortality caused by advanced, metastatic breast cancer. The work proposed will critically test an important, novel pathological role for AMF in vivo.

TUMOR-SECRETED AMF: CAUSAL ROLE IN AN ANIMAL MODEL OF CACHEXIA

Lay Abstract: JM Chirgwin, Ph.D.

Cachexia is a devastating wasting syndrome of advanced cancer and a major contributor to death from metastatic disease. Cancers secrete protein factors which cause muscle and fat wasting and loss of appetite in patients, but progress in identifying such factors has been limited. We recently carried out the first animal experiments with an active cytokine made by tumor cells, called autocrine motility factor [AMF.] We found that cells engineered to secrete mouse AMF caused severe cachexia when grown as intramuscular tumors in nude mice. Mice bearing control cells not secreting the factor developed large tumors but did not lose weight. In 1996 it was discovered that AMF was identical to phosphoglucose isomerase [PGI], which has been studied as a serum marker of advanced metastatic breast cancer for 50 years. We have also observed that mice with bone metastases caused by human breast cancer cells have severe weight loss and increased serum concentrations of AMF/PGI. The results strongly suggest that AMF/PGI secreted by tumor cells is a causal factor in cachexia.

The AMF/PGI molecule is a large protein with a variety of functions: inside the cell it is an essential enzyme in glucose metabolism, phosphoglucose isomerase. Outside of the cell, it is a tumor-secreted factor with actions on nerve and immune cells (neuroleukin), and tumor cells (autocrine motility factor, maturation and differentiation factor). The factor is also associated with the symptoms of rheumatoid arthritis, a disease also causing cachexia. We recently found that tumor cells secreting AMF caused pathological bone formation in two different animal models of bone metastasis -a response similar to what is found with about 15% of breast cancers metastatic to bone. Animals with significantly increased serum AMF concentrations displayed tumor-associated weight loss, a major cause of morbidity and mortality in advanced disease. AMF expression was recently reported to be altered in breast cancer cells by heregulin; so AMF could be altered by clinical treatment with Herceptin.

All of these data suggest that AMF, secreted by tumor cells, and acting on host cells, contributes to the pathology associated with advanced metastatic breast cancer. A difficulty in testing the effects of AMF/PGI in vitro has been inability to regulate its secretion from tumor cells, since this occurs by an unknown mechanism. We have therefore developed procedures for producing pure recombinant protein in sufficient quantity for direct testing in mice. We have cloned, expressed, and purified both mouse and human AMF proteins and determined the x-ray crystal structure of human AMF to high resolution. Our experiments with mouse and human bone cells indicates a 100-fold species preference for mouse versus human factors: to elicit the same response from mouse cells 100X more human than mouse AMF is needed . We propose critically to test the role of AMF/PGI in vivo:

Aim 1: We will administer recombinant mouse AMF/PGI protein by implanted mini-pumps into nude mice to raise plasma concentrations of the factor. Effects on animal weight and serum AMF concentrations will be followed and internal organs assessed at autopsy.

Aim 2: Tumor-secreted AMF retains the enzyme activity of intracellular phosphoglucose isomerase; it is unknown whether this is needed for extracellular functions. We will test the effects on mice of isomerase-inactive AMF mutant protein.

Aim 3: AMF/PGI has species-specific effects on cells in vitro. We will test whether this is also true in vivo. Aim 1 will be repeated with recombinant human AMF/PGI in place of the mouse factor.

Aim 4: The effects of AMF/PGI are the result of binding of the molecule to high-affinity receptors on the surface of target cells, but the details of this binding are not understood. We will use recombinant mouse and human AMF proteins to determine the biochemical mechanism of receptor:ligand binding.

Aim 5: The cachectic actions of AMF could be prevented by blocking ligand:receptor binding. We have raised a panel of monoclonal antibodies against recombinant AMF/PGI. We will test the individual antibodies for their ability to inhibit ligand binding as in Aim 4. Blocking antibodies will be tested in vivo for their ability to decrease cachexia induced by recombinant AMF/PGI as in Aim 1.

The work proposed will provide all of the techniques to screen for effective drugs to block the pathological effects caused by AMF secreted from advanced breast cancers. The actions of AMF in vivo have been previously uninvestigated due to lack of experimental tools. We have now developed the necessary tools to test critically the actions of AMF to cause tumor cachexia and then to develop treatments to inhibit the pathological consequences of increased blood levels of tumor-produced AMF.

TUMOR-SECRETED AMF: CAUSAL ROLE IN AN ANIMAL MODEL OF CACHEXIA

Statement of Work J.M. Chirgwin, Ph.D.

Task 1 (Aim 1) Determine dose range and Alzet mini-pump size for administration of mAMF to achieve effective blood levels of ~10ng/mL. [**28 mice**] Year 01, months 1-6.

Task 2 (Aim 1) Demonstrate cachexia with mouse AMF infused into mice, compared to controls and to mice bearing CHO-K1 [mAMF-] and CHO-1C6 [mAMF+] IM tumors. [**56 mice**] Year 01, months 7-12.

Task 3 (Aim 1) Carry out routine pathology and histology of animals from Task 2. Year 01, months 10-12.

Task 4 (Aim 1) Determine host concentrations of 4 host cytokines in baseline and sacrifice blood samples of animals from task 2. Year 02, months 1-4.

Task 5 (Aim 2) Construct two mutants of mAMF [E357A and 4S/T-A] and sequence. Year 02, months 1-4.

Task 6 (Aim 2) Express and purify mutant mAMF proteins and determine Km and Vmax and binding to phosphocellulose. Year 02, months 5-8.

Task 7 (Aim 2) Carry out Task 2 & 3 protocols with wt & 2 mutant mAMFs by pump infusion. [**24 mice**] Year 01, months 9-2; Year 03, months 1-3.

Task 8 (Aim 3) Carry out Task 2 & 3 protocols with mouse versus 3 concentrations of human AMF. [**40 mice**] Year 02, months 1-12.

Task 9 (Aim 4) Determine Kd and Bmax values for binding of [^{125}I]-mouse AMF to mouse and human cell lines. Determine Kd and Bmax values for binding of [^{125}I]-human AMF to mouse and human cell lines. Identify mouse cell line with high Bmax value. Year 01, months 1-12.

Task 10 (Aim 5) Test ability of 6 different anti-AMF mAbs to inhibit binding of [^{125}I]-mouse AMF to mouse and cell lines in vitro, as in Task 9. Year 02, months 1-6.

Task 11 (Aim 5) Purify specific mouse IgG mAb against AMF, identified in Task 10, from hybridoma supernatant by protein G chromatography[73]. Year 02, months 7-12.

Task 12 (Aim 5) Carry out Task 2 & 3 protocols with mouse AMF and the specific mAb IgG from Task 11 versus control, pre-immune mouse IgG (Sigma). [**32 mice**] Year 03, months 1-12.

Task 13 (Aims 1-5) Analyze data, prepare and submit results for meeting presentations, progress reports, and peer-reviewed publication. Year 01-03, throughout.

Total mice requested = 180 [adult female Balb/c & Balb/c nudes]

TUMOR-SECRETED AMF: CAUSAL ROLE IN AN ANIMAL MODEL OF CACHEXIA

Relevance Statement: JM Chirgwin, Ph.D

A major cause of morbidity and mortality for patients with advanced cancer is cachexia, a paraneoplastic syndrome characterized by severe muscle wasting, loss of appetite and malnutrition[27]. For example, the majority of patients dying from breast cancer have disease metastatic to the skeleton[15]. Bone metastases themselves do not appear to be a major cause of death; patients have a 24 month average survival from time of diagnosis of bone metastases. A major cause of death in these patients is cachexia secondary to tumor in bone[45]. A number of cytokines have been implicated as causal agents in cancer cachexia[35,60]. Tumor cells must secrete factors which either directly[44] cause cachexia in the host or stimulate production of host factors, particularly from the immune system[35], which in turn cause the cachectic response. Despite the serious nature of the clinical problem, only a few tumor-produced cachectic factors have been identified. Phosphoglucose isomerase [PGI] has been known for 50 years[5] as plasma marker of advanced, metastatic breast cancer but was not suspected to contribute functionally to the disease. Five years ago[64,68] PGI was discovered to be identical to the multifunctional cytokine, autocrine motility factor [AMF]. We have established an animal model to test the effects of AMF in bone metastases[32]. We noticed that control experiments which resulted in increased serum concentrations of AMF resulted in profound cachexia in mice. These results suggest that **increased serum concentrations of AMF in patients with advanced breast cancer may cause cachexia**.

It was observed nearly 50 years ago that patients with advanced breast cancer, in particular with metastases to bone, had increased serum activity of phosphoglucose isomerase[5]. Since then, over 200 papers have examined the utility of PGI as a cancer marker, but it seemed to be an indicator of advanced disease and thus of little prognostic utility[70].Since PGI is a ubiquitous intracellular glycolytic enzyme, it was assumed that its presence in the serum of patients indicated cellular lysis, perhaps of necrotic tumor cells. No data were available to address whether elevated circulating PGI levels had *functional consequences* for the patients. The situation changed dramatically in 1996, when the laboratory of Raz[64] found that the amino acid sequence of tumor cell autocrine motility factor was the same as that of PGI, and the group of Chiao found that a factor which induced maturation and differentiation of human leukemic cells was also the same molecule[68]. PGI had been reported earlier to be identical to neuroleukin[21], with effects on neurons and on B lymphocyte maturation. More recently the factor was discovered to be the antigen in a form of autoimmune rheumatoid arthritis[36], a disease often accompanied by cachectic weight loss[37], as well as a major sperm antigen[69]. It has recently been reported that heregulin (whose action is blocked by the clinically effective antibody Herceptin) regulates AMF expression in breast cancer cells[58]. We have found that AMF has potent species-specific effects on bone cells[32]. AMF, as PGI, is an essential intracellular enzyme. Deficiencies cause hemolytic anemia and hydrops fetalis[50]; mice null for PGI are early embryonic lethals[65,66]; so therapies directed at globally blocking the protein itself are impractical.

The extracellular cytokine activities of AMF are mediated through a widely-expressed cell surface receptor, but the actions of AMF in intact animals have not been investigated for several reasons: 1) our data indicate a 100X species-specificity for mouse versus human AMF proteins; 2) AMF is not a conventionally secreted protein, so its expression cannot be regulated from engineered cells; 3) recombinant protein has been unavailable. We have developed an efficient system for the production of large amounts of pure, active recombinant AMF for mouse, rabbit, and human proteins. The human factor has been crystallized and its x-ray structure solved to 1.6Å resolution[50]. We have purified sufficient recombinant mouse AMF to test its cachectic effects in mice. We have the reagents to determine binding of AMF to its receptor and to test anti-AMF monoclonal antibodies for their ability to prevent binding of ligand to receptor in vitro and cachexia in vivo.

The work proposed will critically test: 1) the ability of AMF to cause cachexia in vivo using a mouse model; 2) the species-specificity of mouse versus human AMFs; 3) the mechanism of binding of AMFs to their receptors on the surface of cells; 4) the ability of anti-AMF antibodies to block binding in vitro and to decrease cachexia in vivo. Also tested will be the relation of PGI enzyme activity to AMF receptor-binding activity. If isomerase activity were required for AMF responses, it would be possible to design isomerase inhibitors[12] with extracellular (anti-AMF) but not intracellular (anti-glycolysis) actions. These experiments will open a new avenue to developing therapeutics to inhibit cachexia in advanced breast cancer. Prevention of cancer-induced cachexia would increase survival and quality of life for many patients with metastatic disease.

TUMOR-SECRETED AMF: CAUSAL ROLE IN AN ANIMAL MODEL OF CACHEXIA

Background: Cachexia is a major source of morbidity and mortality for patients with advanced cancers. It has three clinical features: 1) loss of appetite (anorexia), which probably has a central nervous system component, 2) nutritional mal-absorption, and 3) muscle and fat wasting caused by tumor-stimulated factors[27,60]. This application focuses on the 3rd component. A number of factors have been proposed to cause cancer cachexia[35,59]. These fall into two classes: primary ones produced by the cancer cells themselves and secondary ones, which are inflammatory factors released by the host in response to the tumor. The existence of tumor-produced factors has been long known[44], but few such factors have been identified at the molecular level. In addition, cachexia is characteristic of AIDS, rheumatoid arthritis[51], and other diseases, as well as cancer. Despite extensive characterization of cytokine involvement in cachexia, progress in treatment of cancer cachexia has been limited[1,41], and treatments aimed at inhibiting the actions of host-produced inflammatory mediators have not been widely successful. Lack of progress in the area is unfortunate, given the tremendous benefit patients with advanced cancer would receive from effective treatment of cachexia to improve their quality of life and postpone mortality.

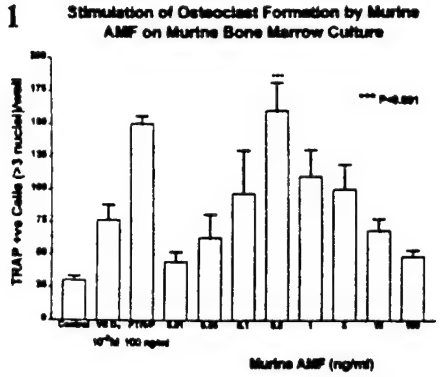
Biochemical mechanisms of cachexia have been explored in vivo and in vitro. One of the major consequences of advanced cancer is severe skeletal muscle wasting[61]. This appears to be due to activation, through characterized pathways, of proteasomal degradation of structural proteins in muscle[22,67]. Less understood are the primary factors released by tumor cells responsible for initiating the muscle wasting.

A variety of **animal models of cachexia** have been described. These fall into two categories. In one type, different cancer cell lines or sublines are compared, with one causing more weight loss than another. A second model is more convincing: expression cDNA is stably expressed in a clonal cell line, often Chinese hamster ovary [CHO]. The cell line, when introduced into nude mice, causes cachexia compared to control cells. In principal the two cell lines differ only by one gene, which therefore should be responsible for the weight loss observed in the animals[35]. Experiments of this type have shown that interleukin [IL]-6[3,17], interferon-γ[34], tumor necrosis factor [TNF]-α[40,74], and transforming growth factor [TGF]-α[71,75] can cause cachexia in this animal model. However, these are probably all host-response factors, rather than primary factors produced by patient tumors. When cachexia-causing tumors were tested in mice genetically modified to be resistant to the actions of several of these inflammatory cytokines, tumor-induced cachexia was not prevented[8]. The data suggest that these factors alone do not account for cancer cachexia. Much of the work cited above on IL-6, TNF-α, and TGF-α, was carried out by colleagues in the P.I.'s Division in San Antonio, in the context of effects of cancers on the skeleton.

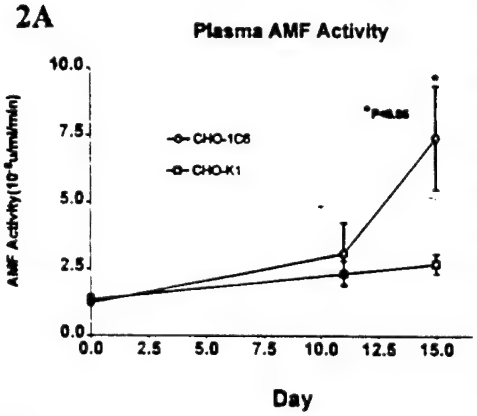
In addition to the known inflammatory factors described in the previous paragraph, there are presently **two characterized tumor-produced factors which appear to cause cachexia** in the host: parathyroid hormone-related protein [**PTHrP**] and proteolysis-inducing factor [**PIF**]. PTHrP is the major causal factor in humoral hypercalcemia of malignancy [HHM][19,20] and osteolytic bone metastases[13]. It has receptor-mediated actions on the kidney and on bone to enhance osteolytic bone destruction. We[73] and others[45] have observed that osteolytic bone metastases are almost invariably accompanied by cachexia. A role for PTHrP in tumor cachexia has not been widely appreciated in the cancer field, beyond investigators focused on bone metastases[45]. For example, a number of the well-studied models of cancer cachexia have been shown in recent years to secrete PTHrP[18,46]. The colo26 colon cancer line causes cachexia in nude mice. It causes HHM consequent to PTHrP secretion, which can be blocked with bone-antiresorptive agents[10] with accompanying prevention of cachexia. Since HHM is characterized by severe elevation in blood calcium concentration and effects on kidney function, it is unclear whether cachexia is a primary or secondary reaction to tumor secretion of PTHrP.

PIF is a peptide purified from urine of cachectic animals[33,62]. When injected into animals the peptide reproduces cachexia. No cDNA sequence for PIF has been published, but a commercial patent [Incyte Pharmaceuticals: Akerblom IE, Murry LK (1998). Human cachexia associated protein. U.S. Patent 5,583,192.] describes a cDNA which includes the reported N-terminal sequence of PIF[62]. This sequence does not give any significant matches in the present Genbank database of human and mouse sequences when subjected to a BLAST search (Chirgwin, unpublished), suggesting that PIF/HCAP may be produced by an opportunistic microorganism. Chlamydial infection, for example, may contribute to cachexia in patients with AIDS, and in general patients with cachexia are immunocompromised.

1



2A



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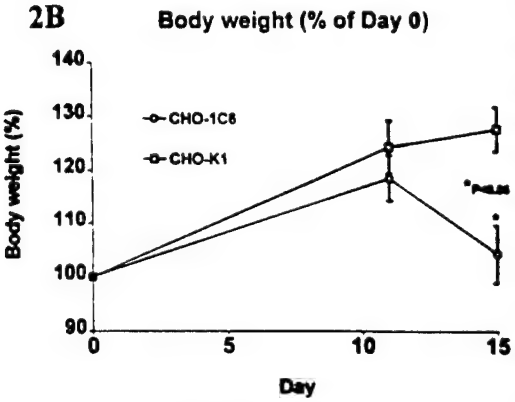

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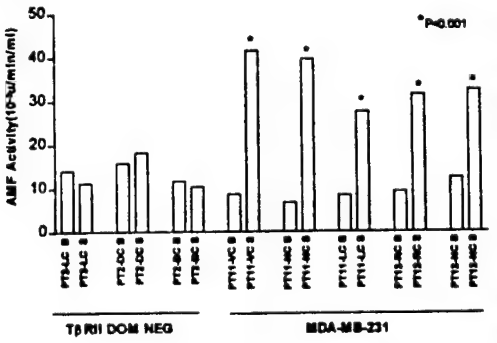

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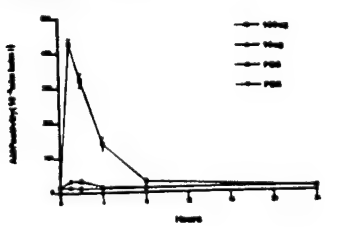

Preliminary data. We initiated work on AMF/PGI with the hypothesis that it contributed to osteolytic bone metastases[15]. The work, funded by an Army IDEA award, was based on the observation that the protein stimulated macrophage differentiation of HL-60 leukemic cells[68]. Since osteoclasts belong to the same cell lineage, we hypothesized that the AMF/PGI protein would stimulate osteoclast formation. This is true in vitro[32]. The response is mediated by the induction of the known osteoclast stimulator, RANK ligand, in bone marrow stromal cells. The response was biphasic (no effect at high or low concentrations, only at intermediate ones around 10nM), which has been observed in all other bioassays (**Figure 1**). **Relation between serum PGI/AMF and cachexia**. Prior to our work, no studies of AMF/PGI actions in vivo had been described, because no experimental system existed for delivery of the factor. Recombinant material was not available and (as explained below), transfection of AMF cDNA into cells does not cause secretion of the factor. For our experiments we made use of the CHO-1C6 cell line - engineered by stepwise gene amplification (an expensive, laborious, year-long process[52]) to overexpress and secrete mouse neuroleukin (which is the same molecule as PGI/AMF). We tested CHO-1C6 versus CHO-K1 controls in nude mice by inoculating 10^5 cells into the thigh muscle. CHO-K1 cells formed somewhat larger tumors, but only the smaller CHO-1C6 tumors caused cachexia. The data are shown in **Figure 2**. We observed that nude mice with bone metastases show a direct correlation between bone lesions, serum AMF/PGI, and weight loss. We described experiments with nude mice bearing osteolytic lesions due to MDA-MB-231 breast cancer cells[73]. Subclones stably transfected with a dominant negative TGFβ receptor had decreased tumor burden and bone lesions and increased survival (ref 73, figures 3&4). Survival of the animals was inversely related to cachexia. We assayed individual animals from these experiments for serum AMF/PGI at baseline and at 4 weeks. Animals carrying the dominant negative receptor had increased survival, no weight loss and no change in AMF/PGI. The animals carrying control MDA tumors had significant weight loss and 2.5X increase in plasma AMF/PGI compared to baseline (**Figure 3**; b=baseline; s=sacrifice). We also injected several animals with 10 or 100μg recombinant mouse AMF/PGI and assayed circulating PGI activity from retroorbital bleeds. The data (**Figure 4**) suggest a serum half-life of approximately 2hrs for the injected protein (Li & Chirgwin, unpublished), with the higher dose giving a very large increase in serum level.

Background on PGI/AMF: **Biological Activities of AMF**: Autocrine motility factor has been periodically rediscovered. All of these activities are caused by the same protein product of the single mammalian gene for phosphoglucose isomerase [**PGI**]. PGI is the first enzyme of the glycolytic pathway, catalyzing the interconversion of glucose 6-phosphate [G6P] and fructose 6-phosphate [F6P][43]. Patients with advanced breast cancer, in particular with metastases to bone, have increased serum PGI activity[5]. Several hundred papers have tested PGI as a cancer marker; it indicates advanced disease and so is of little prognostic utility[2,9,16,38,53,70,72], but the question was long unaddressed of whether elevated circulating PGI levels had *functional consequences* for the patients.

In 1986 Gurney et al. described the sequence of a novel cytokine, neuroleukin, which had potent effects on neuronal survival and on B lymphocyte maturation[21]. This factor was later realized to be identical to PGI.. In 1996 the amino acid sequence of tumor cell autocrine motility factor [**AMF**] was found to be that of PGI[42,64]. AMF stimulates tumor cell motility *in vitro*. Since AMF is the name most commonly encountered in the cancer literature, we use it here. A factor which induced maturation and differentiation of human leukemic cells was also the same molecule[30,68]. PGI is the antigen in a form of autoimmune rheumatoid arthritis[36,37], as well as a major sperm antigen[69]. AMF has effects on breast cancer cell growth and apoptosis[11], and is regulated by heregulin[58].

Nothing is known about the mechanism of release of AMF from cells, except as a consequence of cell lysis. The protein has no signal peptide and does not pass through the ER-Golgi secretory pathway[23,28]. This nonclassical secretory pathway may be used by other factors such as IL-1 and FGFs 1 and 2, but it remains very poorly understood. A serious consequence of this pathway is that there is no known methodology for controlling cellular secretion of AMF/PGI. Thus, stable transfection of AMF cDNA into mammalian cells results in increased cytoplasmic PGI enzyme activity but not secretion of AMF outside of the cell (unpublished). We found that AMF has potent, species-specific effects on bone cells[32]. In vitro it induced expression of the bone-resorptive cytokine, RANK ligand, on stromal cells. In two animal models AMF caused disorganized new bone formation in vivo, characteristic of the osteoblastic lesions seen with 15% of breast cancers metastatic to bone. Partially inactivating mutations in PGI cause hemolytic anemia and hydrops fetalis[50], while mice homozygous for PGI null alleles are early embryonic lethals[65,66]. Thus, systemic therapies directed at blocking the expression or enzymatic activity of the PGI/AMF protein are impractical, since they would block glycolysis in all cells.

PGI/AMF Protein Structure & Expression: The recent revival of interest in the protein has resulted in the publication of x-ray crystal structures of PGI from *Bacillus stearothermophilus*[14,57] and from rabbit muscle[24,25]. The structures from these divergent sources show a highly conserved core surrounding the active site. We have collaborated with Christopher Davies, Univ of Sussex, Brighton UK to complete an old rabbit muscle crystal structure bound with the competitive inhibitor, 5-phosphoarabinonate[12]. To this end we cloned, sequenced and expressed the rabbit enzyme[31]. We developed a system for expression and purification of recombinant protein in *E. coli*, described in this paper. A $(His)_6$ C-terminal extension permits one-step purification to >95% purity of active, LPS-free enzyme. A liter of bacterial culture yields ~50mg of recombinant protein. We have extended this approach to the mouse and human proteins (both cloned from available IMAGE DNAs). Parallel results have been obtained (Li, Hill, Chirgwin, in preparation). The mouse and human proteins crystallize readily, and the X-ray structure of the latter has quickly been solved to 1.6Å[50]. The biological activity of these preparations is identical to that of the naturally occurring proteins (Schultz, Hill & Chirgwin, in preparation).

Various references have been made in the literature to activities (AMF and neuroleukin-like) of bacterial PGI on mammalian cells[14,57] and of similar activities of undefined fragments of human AMF protein[11,42]. PGI is a dimer of 125kDa, which can only be dissociated by treatment with 5M guanidine HCl[4]. Removal of denaturant results in dimer reformation or aggregation[7]. It seems clear that active mammalian PGI has the extracellular activities attributed to it. It is certainly possible that proteolytic fragments of PGI/AMF[56] could bind efficiently to the AMFR, but we do not believe that any of the published claims for bacterial or fragment bioactivities are based on sufficient biochemical evidence to be persuasive[50].

*AMF receptors [**AMFR**]*: All of the extracellular effects of AMF are probably mediated through a widely-expressed cell surface receptor. A cDNA was first identified[63] using a putative anti-receptor rat monoclonal IgM antibody 3F3A[39]. The receptor was incorrectly described as having a single transmembrane domain and homology to the nuclear p53 tumor suppressor protein. Drastically revised sequences of mouse and human AMFRs reported[54] that the human sequence was partly the same as that of Watanabe et al[63]. The AMFRs now encode seven-transmembrane G-protein coupled receptors. They are not close relatives of known family members and have very large cytoplasmic domains. No data have been published to demonstrate that they bind AMF ligand. The receptor antibody has agonist activity (gives an AMF response *in vitro* in the absence of ligand). Data with the antibody[55] suggest the receptor signals through protein kinase C. Recent analysis suggests that the putative AMFR is an intracellular protein resident in the endoplasmic reticulum[49]. Recently Le et al[29] demonstrated co-localization of cellular staining with anti-receptor antibody and with biotinylated rabbit AMF. The data suggest that the 3F3A antibody does recognize an authentic receptor for AMF, but the cDNA reported[54] may not encode the correct protein.

Objectives & Specific Aims, along with Design Rationales::

1) **Is recombinant AMF protein sufficient to cause cachexia in vivo?** Recombinant mouse AMF will be delivered continuously to mice via Alzet mini-pumps. The first experiment will determine the dosage to achieve blood levels equivalent to those seen in mice with tumor-induced cachexia. Pure recombinant mouse AMF will be infused, eliminating any confounding effects of unknown factors possibly secreted by the CHO cells. AMF in serum will be determined by assay of 10μl blood samples. AMF may induce host inflammatory cytokines. We will assay for cachexia-associated cytokines, IL-1, IL-6, IFN-γ,and TNFα, in the sera of AMF-treated mice.

2) **Is isomerase catalytic activity required for AMF-induced cachexia?** Inside the cell PGI interconverts glucose and fructose 6-phosphates in the glycolytic pathway. It is unclear if enzymatic activity is needed for the extracellular cytokine activities of the protein. We will make 2 mutants of AMF/PGI: E357A, which lacks the side chain responsible for catalytic proton transfer[25,50], and S209T211,214,217/4A, which eliminates the groups needed to bind the phosphate group of the substrate. Experiments will be guided by the 1.6Å x-ray structure of the human enzyme[50]. Mutant proteins will be assayed in vitro for isomerase activity and receptor binding and for ability to cause cachexia as in Aim 1.

3) **Are the cachectic actions of AMF species-specific?** Our data with bone cells in vitro show a 100-fold preference for AMF of the same species when mouse and human are compared -predicting that much higher concentrations of human AMF than mouse AMF will be required to cause cachexia in mice, as in Aim 1.

4) **Does AMF act via a high-affinity cell-surface receptor?** AMF shows a bell-shaped dose response curve in vitro and in vivo, suggesting an activation mechanism in which receptor is dimerized by low concentrations of dimeric ligand. We will test this mechanism by biochemical binding studies with [^{125}I] mouse and human AMF ligand binding studies to mouse and human cell lines in vitro.

5) **Can cachexia be alleviated by treatment with monoclonal antibodies which prevent AMF binding to its receptor?** We have raised a panel of monoclonal antibodies [mAbs] against recombinant AMF/PGI. We will test the individual antibodies for their ability to inhibit ligand binding in vitro as in Aim 4. Blocking antibodies will be tested in vivo, if time permits, for their ability to decrease cachexia induced by recombinant AMF as in Aim 1.

Methods: *General methods*: All molecular biology and protein chemistry is by standard means and has been recently described[31]. PGI/AMF is determined by coupled spectrophotometric assay[43], in which the dilute, rate-limiting isomerase converts F6P to G6P, which is instantly consumed by excess G6PDH (1 unit yeast enzyme, Sigma), and the formation of NADPH cofactor from NADP+ is followed at 340nm. Reaction volume is 250μl in a 37°C-thermostatted Cary 219 recording spectrophotometer. The method can accurately detect less than 0.1 ng/ml PGI with a 10μl sample. Sensitivity is limited only by yeast PGI contamination of the coupling G6PDH enzyme. A Sigma kit is also available for the determination of PGI. The concentration of PGI/AMF in baseline serum samples from nude mice is 1-2ng/ml. 1ng/ml is equivalent to 8pM of 125kDa dimeric protein.

Aim 1) Is recombinant AMF protein sufficient to cause cachexia in vivo? *Preparation of AMFs*: Li & Chirgwin[31] describes the cloning, expression and purification of rabbit AMFH_6 as a 1.7kb Nde I to EcoR I restriction fragment in the bacterial expression vector pET5a, in which the start codon is encoded by the Nde I site and the original stop codon is replaced by 6 histidine codons, followed by a new UAA stop codon and an EcoR I site. This vector permits expression in the *E. coli* host BL21DE3pLysS, induced with iPTG to yield ~50mg AMF per liter of culture. Vector and host are from Stratagene and used according to the supplied instructions. Cells are lysed and the His-tagged protein (564 aa) purified to greater than 95% homogeneity (single 63kDa band on denaturing, reducing PAGE gel stained with Coomassie blue) in one step by batch chromatography on NiNTA agarose (Qiagen) according to the manufacturer's standard protocol. We made parallel constructs of mouse and human AMFH_6s from EST clones [IMAGE consortium]. Both cDNAs gave abundant PGI activity in mammalian cells (expressed in pcDNA3 vector) and in the pET5a system. The purified proteins have kinetic parameters very similar to those of the rabbit enzyme[43] and have produced excellent quality crystals for x-ray analysis (human[50]; mouse-C. Davies, personal communication.) NiNTA-purified proteins were devoid of endotoxin/LPS contamination as determined by Sigma kit. *Ligand delivery:* mouse (or human in Aim 3) AMFs are concentrated with centricon 30 centrifugal dialyzer-concentrators to 20mg/ml in PBS and 0.2μ filter-sterilized. This solution (200μl) will be placed sterilely into Alzet 2000, 2001 and 2001D minipumps [www.alza.com/alzet/] which deliver 0.2, 1, or 8μl/hr. One set of 2000s will be filled with 2mg/ml instead of 20. Pumps will be placed subcutaneously on the dorsal aspect under

anesthesia and the incision clipped. Serum AMF levels will be determined by PGI spectrophotometric assay[43] on 10μl aliquots of retroorbital blood, taken at baseline and at 1 hr intervals for 8 hrs after implantation and at 24 hrs and daily thereafter. This experiment is designed to determine the appropriate dosing protocol to achieve ~4X elevation of serum PGI/AMF 95-10ng/ml) compared to baseline. We will use 4 female adult Balb/c mice per group, four groups receiving pumps with AMF and a 5th group pumps with PBS. Number of animals = 20. AMF delivery rates will be 0.4, 4, 20, and 160 μg/hr. From the initial experiment, we will choose an appropriate pump size and AMF loading concentration. This dose regimen will be retested with 4 mice per group, one experimental and one sham group (8 animals). *Main animal experiment*:will use 8 animals per group with Balb/c nude females: group 1-no treatment; group 2-CHO-K1cells (10^5) IM; group 3-CHO-1C6 cells (10^5) IM; group 4) minipumps with PBS; group 5 minipumps with mouse AMF. Weight-matched Balb/c's (non-nude) will duplicate groups 3 and 4 to test whether subsequent experiments can be done with immunocompetent mice. Nudes need to be used initially to replicate the original CHO cell data as an essential positive control. Animals will be followed for up to 4 weeks. Previously CHO-1C6 animals had to be sacrificed at 15 days due to severe cachexia. Pumps will be replaced as recommended by the manufacturer (at one week intervals for model 2001s). Animals will be weighed daily and monitored for paraplegia and lethargy. Animals will be sacrificed at 28 days or when cachectic or paraplegic by CO2 asphyxia and exsanguinated for subsequent blood analyses for AMF, whole blood ionized Ca [to exclude cachexia consequent to HHM], and cachectic factors. Animals will be subjected to standard necropsy inspection. Livers, spleens, and kidneys will be weighed, fixed, and stained with H&E and examined microscopically. *Does AMF activate host genes associated with cachexia?* AMF causes differentiation of monocytic cell lines *in vitro*[68], a consequence of which is the induction of interleukin 1 secretion. IL-1 is a potent inflammatory factor, which suggests a possible mechanistic relationship between elevated serum AMF levels and tumor-associated cachexia. We will test the ability of AMF to induce cachexia-associated cytokines IL-1β, IL-6, IFN-γ, and TNFα Serum samples will be collected from the mice at baseline and sacrifice and assayed in triplicate using mouse-specific ELISA kits from R&D Systems according to the manufacturer's instructions. We tested blood samples at sacrifice from several cachectic mice bearing CHO-1C6 IM tumors. The samples contained no detectable mouse IL-6 or TNFα (Li & Chirgwin, unpublished), but this work needs to be repeated.

Aim 2) Is isomerase catalytic activity required for receptor responses? It has been claimed that a bacterial phosphoglucose isomerase has AMF activity and that AMF function requires isomerase activity, but the data are unconvincing. Bacterial protein with unreported LPS content was used in mammalian cell assays, and the data lacked controls or statistical analysis of significance[14,57]. Experiments with competitive inhibitors of isomerase activity are also unpersuasive[57]. The best of these inhibitors, developed by the P.I.[12,25], is effective only at a concentration in a million-fold excess over the AMF ligand. A rigorous test of the relationship between PGI enzymatic activity and AMF receptor-mediated bioactivities is needed. We will make two mutants at the PGI active site by standard DNA mutagenesis in vitro and test them exactly as described in Aim 1. PGI residue E357 is responsible for catalytic proton transfer between C1 and C2 in the enediolate-mediated isomerization[25,50]. We will test the E357A mutant for residual isomerase activity and its ability to bind to phosphocellulose followed by substrate elution, according to the elegant procedure of Phillips et al[47]. This will evaluate preservation of the sugar phosphate binding site in the absence of catalysis of isomerization. We will generate a more complex S209T211,214,217/4A mutant, which eliminates the groups needed to bind the phosphate group of the substrate. These residues are shown in Figs 5-7 of ref 50, which illustrate part of the active site of human PGI, with sulfate occupying the phosphate binding pocket. The mutant, abbreviated 4S/T-A, should eliminate substrate binding as well as catalysis, without destabilizing the protein structure. We believe it likely that AMFR activation is not a function of isomerase activity, but the two apparently unrelated functions of this moonlighting protein[23] could be structurally intertwined. The animal experiment will have four groups with implanted minipumps: a) PBS-filled control; b) wt mouse AMF; c) mAMF-E357A; d) mAMF-4S/T-A.

Aim 3) Are the cachectic actions of AMF species-specific? Our data with bone cells in vitro show a 100-fold preference for AMF of the same species when mouse and human are compared- predicting that much higher concentrations of human AMF than mouse AMF will be required to cause cachexia in mice. We will test this as in Aim 1. We will load minipumps with 1X , 10X, and (if practical) 100X the amount of human AMF compared to the effective amount of mouse AMF from Aim 1. There will be five 8-animal groups: PBS, mAMF, and 3 doese

of hAMF. Mouse and human PGI specific activities are indistinguishable by isomerase activity assay of serum.

Aim 4) Does AMF act via a high-affinity cell-surface receptor? AMF shows a bell-shaped dose response curve *in vitro*, suggesting an activation mechanism in which the receptor is dimerized by low concentrations [pM] of the ligand (an obligate dimer). At saturating concentrations, each receptor is bound by a separate AMF molecule; so receptor dimerization is opposed[26]. *Binding analyses*: Confluent monolayers of the various cell lines (we are testing mouse ST2 and human MG63 bone marrow cells) will be incubated with labeled ligand for 2 hours in 1% PBS+BSA on ice to prevent internalization of receptor:ligand complexes. The mouse and human proteins have been iodinated with a commercial kit (Pierce) and [^{125}I] from Dupont-NEN. Labeled protein was purified by spin-column and assayed for radio-specific activity (1-2x10^7cpm/μg) and retention of complete PGI activity to assure lack of protein damage from the iodination procedure. We have similarly prepared active, biotinylated PGI, as has been done by others[29]. Unlabeled mAMF and hAMF will be used as competitors. After extensive washing, the cells will be lysed and bound ligand determined by radioactive gamma counting. Data will be analyzed by the method of Scatchard[48]. Our biological assays show maximum responses of mouse cells to 1-5pM mAMF. Human cell responses require 100X more mouse factor. We anticipate that these responses will reflect the relative affinities of mouse and human receptors for their cognate ligands. Each point will be carried out with n=4 in 96-well dishes. Statistics will be analyzed with InStat software. The P.I.'s first publication utilized these techniques[6]. We have already prepared a series of mouse:human chimeric proteins which are expressed in E. coli and retain enzymatic activity. These could be used in future studies to map the ligand domains which confer species-specificity of binding.

Aim 5) Can cachexia be alleviated by treatment with monoclonal antibodies which prevent AMF binding to its receptor? We have raised a panel of monoclonal antibodies [mAbs] against recombinant AMF/PGI. Human $AMFH_6$ was used to immunize the mice and screen the IgG clones. We identified 3 high titer (hybridoma supernatants + at 1:16,000) clones [11E3, 7G12, 1A7], 2 moderate titer [10H11 & 1H8, + at 1:512], and 1 low titer [9F0, + at 1:64] clones. When these were tested for titer against mouse $AMFH_6$, none showed strong species-specificity, with titers reduced no more than 4X. We will test the 6 hybridoma supernatants for ability to inhibit the binding of [^{125}I]mAMF to mouse cells, as described in the previous Aim. If any of the mAbs are effective, we will purify the mAb and test it in vivo, as we have described previously[73], as time and funds permit. This experiment would use 4 groups of 8 animals: no treatment, mAMF by mini-pump, mAMF + control mouse IgG (Sigma), and mAMF + anti-AMF IgG (2x/wk ip injection, 250μg/injection, based on previous experience[73]).

Animal usage: Aim 1: pilot experiment: 28 mice: main experiment: 56 mice [7 groups of 8 mice each]. Aim 2: 24 mice [3 groups of 8]. Aim 3: 40 mice [5 groups of 8]. Aim 5: 32 mice [4 groups of 8]. Number of mice per group is based on power analysis based on previous animal model experience[73]. Statistical methods for data analysis are also contained in this publication. Total number of animals =180 mice. Until Aim1 is complete, the relative distribution of Balb/c versus nudes for the 180 mice proposed is not predictable.

Innovation: Our preliminary data strongly support the hypothesis that AMF/PGI causes cachexia. Unlike previously identified cachectic factors, AMF has long been known to be elevated in patients with advanced metastatic breast cancer[5]. Because of its nonclassical secretory mechanism, and the fact that homozygous deficient (knock-out) mice are early embryonic lethals, AMF had not previously been tested for its actions in vivo. Our preliminary data are the first to identify a mouse versus human species-specificity and then to test mouse AMF in a mouse model using an engineered CHO cell line. This CHO approach is extremely cumbersome; so we have devoted substantial effort to developing efficient protocols for expressing and purifying recombinant mouse and human AMFs and generated monoclonal antibodies against the latter. We have also solved and published a high resolution X-ray crystal structure of human AMF. These tools are essential to the work proposed. It appears that the extracellular cytokine actions of AMF are independent of the essential intracellular role of PGI in glycolysis. The extracellular actions are receptor-mediated and thus could be inhibited (by antibodies or small molecule receptor antagonists), alleviating the cachectic effects of AMF. Aim 4 will provide the basis for future high-throughput screening for such inhibitors. Prevention of the major paraneoplastic complication of bone metastasis, cachexia, could substantially reduce mortality and morbidity in patients with breast cancer metastatic to the skeleton. Inhibition of cachexia caused by release of AMF from metastatic breast cancers is an experimentally feasible goal and would provide a new therapy to improve quality of life for patients with advanced disease.

TUMOR-SECRETED AUTOCRINE MOTILITY FACTOR: CAUSAL ROLE IN AN ANIMAL MODEL OF CACHEXIA

List of Abbreviations:

AMF	Autocrine motility factor
AMFR	Autocrine motility factor receptor
BSA	Bovine serum albumin
CHO	Chinese hamster ovary
DH	Dehydogenase
ER	Endoplasmic reticulum
FGF	Fibroblast growth factor
F6P	Fructose 6-phosphate
G6P	Glucose 6-phosphate
h	Human
H&E	Hematoxylin & eosin histological stain
HHM	humoral hypercalcemia of malignancy
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
IM	Intramuscular
ip	Intraperitoneal
kb	Kilobases
kDa	Kilodaltons
LPS	Lipopolysaccharide
m	Mouse
mAb	Monoclonal antibody
NADP+	nicotinamide adenine dinucleotide phosphate, oxidized
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
PAGE	Polyacrylamide gel electrophoresis
PGI	Phosphoglucose isomerase
PIF	proteolysis-inducing factor
PBS	phosphate-buffered saline
PTHrP	parathyroid hormone-related protein
R	Receptor
RANK	Receptor activating NF kappaB
SQ	Subcutaneous
TGF	Transforming growth factor
TNF	Tumor necrosis factor

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